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THE PAPILIONACEOUS ALKALOIDS

XVII. THE SYNTHESIS OF STRUCTURAL ISOMERS OF SPARTEINE[1]

BY EDWARD LEETE[2] AND LÉO MARION

ABSTRACT

A structural isomer of sparteine, ψ-sparteine, has been synthesized from 2,2′-dipyridylmethane which was condensed via its lithium salt with β-bromomethylmalonic ester, followed by fission of the ether link. The product of the condensation, αα-di(2-pyridyl)-γγ-dicarbethoxy-propane, was reduced catalytically to the corresponding dipiperidyl compound which on heating *in vacuo* readily lost ethanol, giving rise to two isomeric dilactams. Each dilactam was reducible to a different isomer of sparteine. Just like sparteine, ψ-sparteine can exist in three stereoisomeric forms, two of which, however, are internally compensated and therefore not resolvable.

The stereochemistry of the sparteine alkaloids has been elucidated recently (1, 6, 9) and it has been possible to assign spatial configurations to those alkaloids which are related to sparteine, i.e., anagyrine and lupanine (9), genisteine or α-isosparteine (8), thermopsine (2), and α-isolupanine (9, 10). However, several alkaloids have been reported with the empirical formulae $C_{15}H_{20}ON_2$ and $C_{15}H_{24}ON_2$ in excess of the predicted stereoisomers of anagyrine and lupanine. Such are, for instance, leontidine (17) and sophoramine (11), leontine (17), sophocarpine (12), and matrine (5). It is, therefore, necessary to consider structural isomers of sparteine besides the stereoisomers.

The synthesis of 8-oxysparteine by Anet, Hughes, and Ritchie (1) from 5-aminopentanal, acetone dicarboxylic acid, and formaldehyde under "physiological conditions" (according to Scheme *A*) suggested that a structural isomer of sparteine (I) might equally well form in some plants (according to Scheme *B*). This suggestion is based on the assumption that the actual synthesis of sparteine and its congeners in the plant proceeds according to Scheme *A*, an assumption which still lacks experimental support and is weakened by the fact that Scheme *A* is not exclusive as indicated by Schöpf's recent synthesis of matridine (13).

The structural isomer of sparteine I, which conveniently may be called ψ-sparteine, has now been synthesized from 2,2′-dipyridylmethane II. Attempts were made to obtain this initial substance by the condensation of α-picolyllithium with α-bromopyridine in ether, but very poor yields of the desired product were obtained, the bromopyridine being readily hydrolyzed to α-pyridone

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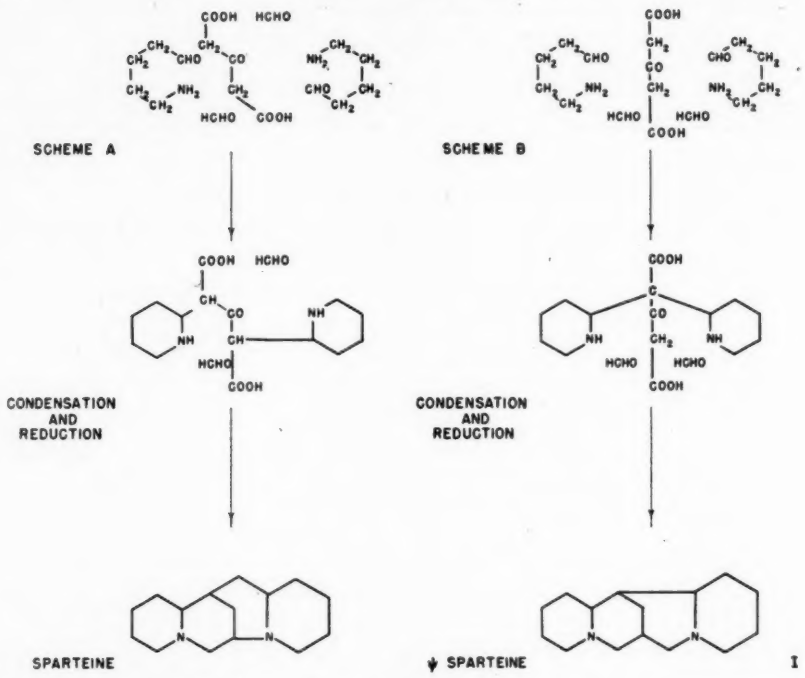
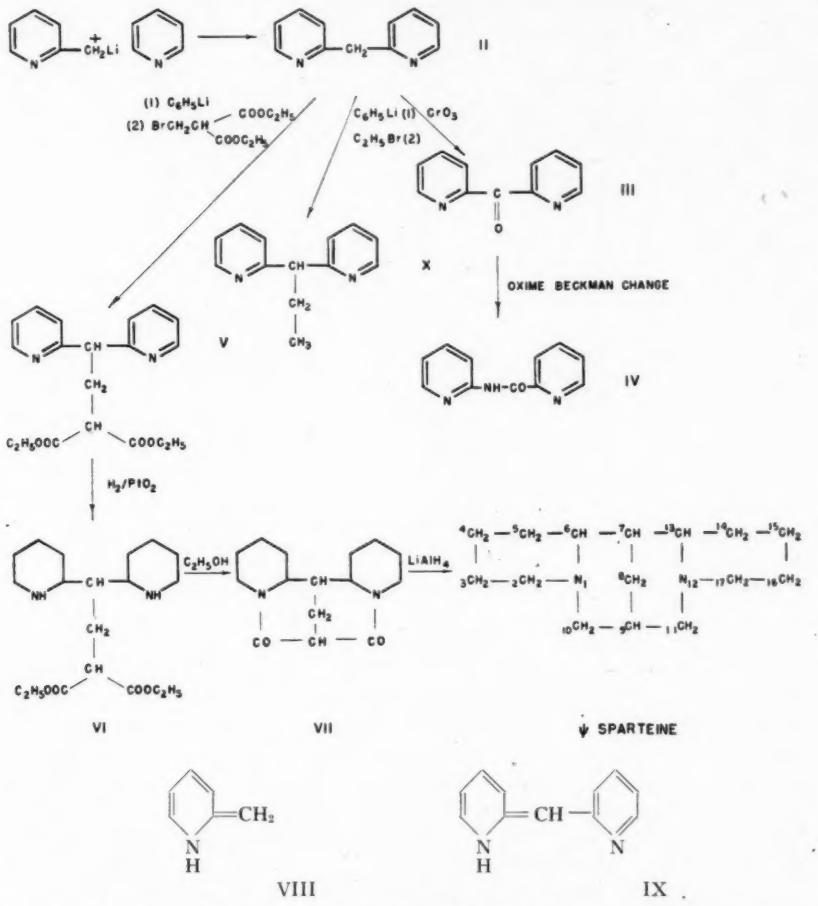
Contribution from the Division of Chemistry, National Research Council, Ottawa, Canada. Issued as N.R.C. No. 2766.
[2] *Holder of a Postgraduate Travelling Fellowship from the Goldsmiths' Company of London, England.*



with the resulting formation of much high-boiling material. A more successful method was the nucleophilic attack of pyridine by α-picolyl-lithium in toluene at 110° in an atmosphere of dry nitrogen, similar to the preparation of 2-phenylpyridine from pyridine and phenyl-lithium (3). The crude dipyridylmethane was purified by fractional crystallization of its hydrobromide. Pure 2,2′-dipyridylmethane was a colorless mobile oil which readily became yellow on exposure to air. It formed a dipicrate and a dihydrobromide. That it possessed the correct structure was confirmed by oxidation with chromium trioxide in acetic acid. The product of the oxidation was di-2-pyridylketone (III), the oxime of which was subjected to the Beckmann rearrangement by the action of phosphorus pentachloride in ether. The amide thus produced, 2-pyridyl-2′-picolinamide (IV), was identical with a specimen prepared from 2-aminopyridine and picolinoyl chloride.

A comparison of the ultraviolet absorption spectra of pyridine, α-picoline, and 2,2′-dipyridylmethane (Fig. 1) shows that α-picoline and dipyridylmethane have very similar spectra, the whole absorption being shifted to longer wave lengths relatively to pyridine. This may be due to the contribution of such structures as VIII and IX which can be brought about by a tautomerism that is not possible in pyridine.

2,2′-Dipyridylmethane like α-picoline gave a lithium salt when its solution in ether was treated with phenyl-lithium. The lithium salt was deep orange in



VIII IX

color and less soluble in ether than the α-picoline salt. In a model experiment the condensation of the lithium salt with ethyl bromide gave rise to αα-di-(2-pyridyl)-propane (X). β-Bromomethylmalonic ester was obtained by essentially the same method as that used by Simonsen (14), i.e., by the condensation of chlorodimethyl ether with sodiomalonic ester followed by fission of the ether link with hydrobromic acid to give the bromo derivative. Analysis of the product indicated that it contained 85% of the bromo compound, the main impurity probably being the unsaturated ester, ethyl methylenemalonate, produced by loss of hydrogen bromide. The bromo ester was condensed with the lithium salt of 2,2′-dipyridylmethane to give αα-di-(2-pyridyl)-γγ-dicarbethoxypropane (V) in 50% yield, the rest of the dipyridylmethane being recovered unchanged. The diethyl ester was a high-boiling viscous oil which could not be distilled without decomposition; hence, the residue left after removal of the unchanged dipyridyl-

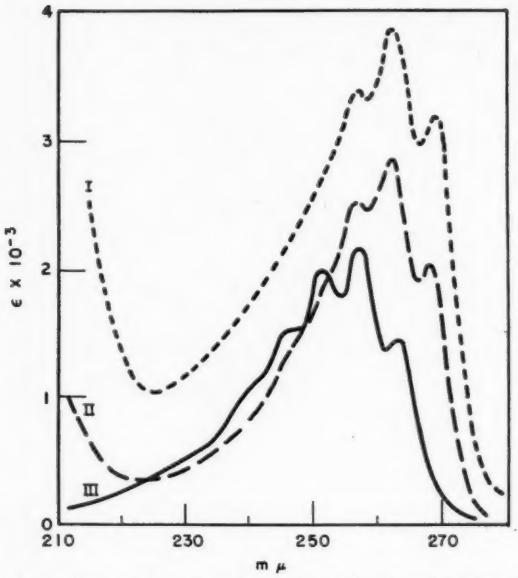


FIG. 1. Ultraviolet absorption spectra taken in 95% ethanol with a Beckman quartz spectrophotometer.
I. 2,2′-dipyridylmethane (ε/2).
II. α-Picoline.
III. Pyridine.

methane by distillation was used in the next stage of the synthesis without further purification. The diester was only weakly basic and no picrate could be obtained; potentiometric titration indicated that the pK value of the most basic nitrogen was less than 3. This reduction in basic strength was presumably due to the presence of the electron attracting ester groups.

Attempts to reduce the diester by the Bouveault method were unsuccessful, but the reduction went smoothly by the action of hydrogen in the presence of Adams' catalyst at 50 lb. per sq. in. No attempt was made to isolate the pure dipiperidyl derivative (VI) since it was found that it readily lost ethyl alcohol on heating *in vacuo*, ring closure taking place with formation of the dilactam (VII). Two diastereoisomeric dilactams were obtained as a result of ring closure, and it was possible to separate them by chromatography on alumina and by crystallization. The two isomers melting at 262-3° (Dilactam *A*) and at 161-2° (Dilactam *B*) were neutral substances. Their infrared absorption spectra, reproduced in Fig. 2, are similar but show distinct differences.

The dilactams were reduced in dioxan with lithium aluminum hydride to give the two ψ-sparteines *A* and *B* as colorless oils which, like sparteine, became yellow and tarry on exposure to air and light. The ψ-sparteines had physical properties very similar to those of the isomeric sparteines. The infrared spectra of the ψ-sparteines and of sparteine, α-isosparteine, and β-isosparteine[3] are shown in Fig. 3.

[3] *β-Isosparteine will be described in a forthcoming publication.*

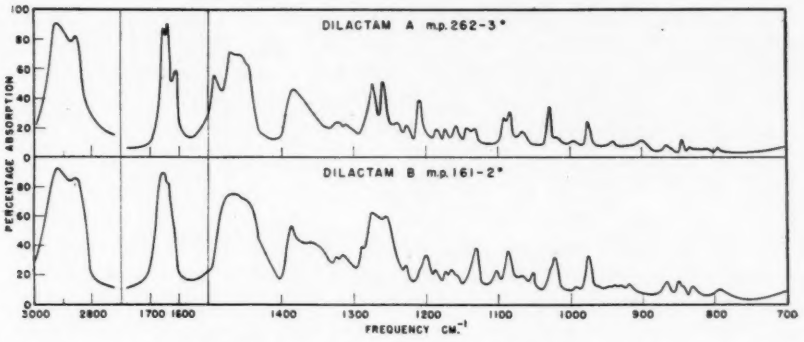


FIG. 2. Infrared absorption spectra measured as nujol mulls on Perkin-Elmer 12B single beam spectrometer with sodium chloride prism.

The stereochemistry of ψ-sparteine is similar to that of sparteine, and theoretically three forms are possible in which the hydrogen atoms on carbon atoms 6 and 13 are either both *cis* (X), both *trans* (XI), or one *cis* and the other *trans* (XII) to the central methylene bridge between carbon atoms 7 and 9. Hence, the stereochemical configuration of compound XII is similar to that of sparteine

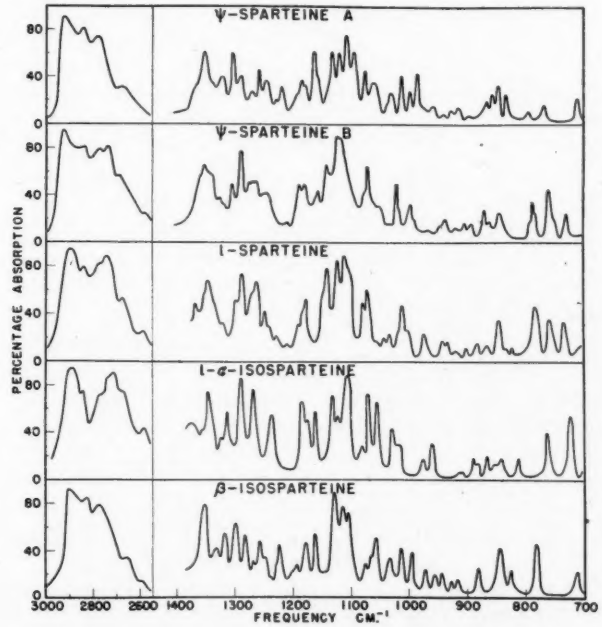
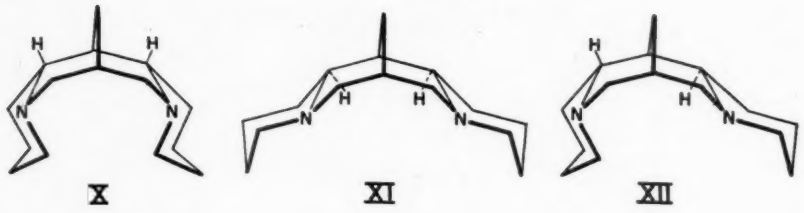


FIG. 3. Infrared absorption spectra measured on Perkin-Elmer 12B single beam spectrometer with sodium chloride prism, 1 mm. cell. Substances in carbon disulphide solution, 10 mgm. per ml.



while the configurations of compounds X and XI are similar to those of α- and β-isosparteines respectively. In contrast, however, to the isosparteines structures X and XI both have a plane of symmetry through carbons 7, 8, and 9 and are internally compensated so that they will not exist as optical enantiomorphs. Structure XII only is asymmetric, its mirror image not being superimposable.

Although *dl*-sparteine has been resolved readily by means of *d*- and *l*-β-camphorsulphonic acids (7), it has not been possible to resolve ψ-sparteine *A* or *B* either with those acids or with 6,6′-dinitro-2,2′-diphenic acid. However, examination of the infrared absorption spectra of the isomeric dilactams in the region of the carbonyl frequencies (Fig. 4) seems to indicate that the two carbonyl groups are not equivalent especially in Dilactam *A* since they apparently

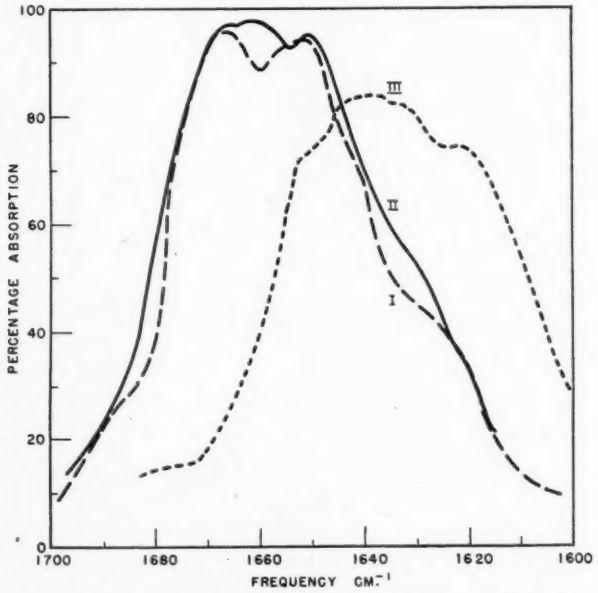


FIG. 4. Infrared absorption spectra measured on Perkin-Elmer 12B single beam spectrometer with calcium fluoride prism, 1 mm. cell. Substances in chloroform solution, 3 mgm. per ml.

I. Dilactam *A*.
II. Dilactam *B*.
III. 10-Oxosparteine.

give rise to absorption peaks at slightly different frequencies. (The infrared spectrum of 10-oxosparteine is also given in Fig. 4 for comparison.) Furthermore in their synthesis of sparteine, Leonard and Beyler (6) obtained sparteine and α-isosparteine, but no β-isosparteine. Hence, notwithstanding the failure so far to resolve the ψ-sparteines it seems more probable that ψ-sparteine *A* is represented by XII whereas ψ-sparteine *B* is either X or XI.

EXPERIMENTAL[4]

2,2′-Dipyridylmethane

Phenyl lithium was prepared by dropping bromobenzene (130 cc., 1.3 mole) in dry ether (150 cc.) on to lithium chips (16.5 gm., 2.4 mole) suspended in ether (600 cc.), in an atmosphere of nitrogen. After stirring for two hours all the lithium had dissolved and a solution of α-picoline (93 gm., 1.0 mole) in ether (100 cc.) was slowly added. Stirring was continued for one-half hour during which the mixture became orange red in color. A solution of pyridine (79 gm., 1.0 mole) in toluene (400 cc.) was then added and the mixture heated to 110° during which the ether distilled off. The reaction mixture was stirred for eight hours at 110° and allowed to cool overnight. It was then further cooled to 0° and water cautiously added. After the lithium hydride had decomposed, hydrochloric acid was added until the liquor was acidic. The aqueous layer was separated, alkalized with potassium carbonate, and the brown oil which had separated was extracted with chloroform. The extract was dried and distilled on the steam bath to remove the chloroform. There was left a residue (161 gm.) which on distillation *in vacuo* gave the following fractions: (i) b.p. up to 100° at 0.5 mm. consisting of α-picoline and pyridine, 37 gm., (ii) b.p. 100-130° at 0.5 mm. crude dipyridylmethane, 62.4 gm., a pale yellow fairly mobile oil, n_D^{20} 1.5872, (iii) a dark brown residue which set on cooling to a very viscous resin. Fraction (ii) was dissolved in 48% hydrobromic acid (80 cc.) and the solution diluted with absolute ethanol when the crude 2,2′-dipyridylmethane dihydrobromide separated (35 gm.). Dilution of the mother liquor with ether precipitated a further quantity of the salt. Fractional crystallization produced the pure dihydrobromide as long colorless needles (27 gm.), m.p. 275-6°. Calculated for $C_{11}H_{12}N_2Br_2$: C, 39.78; H, 3.64; N, 8.44%. Found: C, 39.75, 39.64; H, 3.58, 3.76; N, 8.19%. The pure salt was dissolved in water, the solution alkalized with potassium carbonate, and the oily base extracted with chloroform. The extract was dried, distilled on the steam bath to remove the solvent, and the residual oily base distilled *in vacuo*. 2,2′-Dipyridylmethane thus obtained consisted of a colorless, almost odorless mobile oil, b.p. 106-110° at 0.5 mm. (yield, 12.5 gm., n_D^{20} 1.5759) which rapidly became yellow on exposure to air. It was soluble in water but was salted out by the addition of potassium carbonate; it was completely miscible with the ordinary organic solvents. Calculated for $C_{11}H_{10}N_2$: C, 77.62; H, 5.92; N, 16.46%. Found: C, 77.94; H, 5.97; N, 16.50%. Potentiometric titration in 50% methanol gave pK values of 4.24 and 2.24.

A small quantity of the base in hot ethanol was added to a solution of two equivalents of picric acid in the same solvent. The sparingly soluble dipicrate

[4] *All melting points are corrected.*

which separated immediately was recrystallized from aqueous ethanol from which it separated as small yellow prisms, m.p. 207.5-208°. Calculated for $C_{11}H_{10}N_2 \cdot 2C_6H_3O_7N_3$: C, 43.96; H, 2.57; N, 17.83%. Found C, 43.91; H, 2.57; N, 17.67%. Attempts to prepare a monopicrate were unsuccessful.

2,2′-Dipyridylketone

2,2′-Dipyridylmethane (1.7 gm., 0.01 mole) was dissolved in acetic acid (20 cc.) and to the solution was added chromium trioxide (1.65 gm., 0.0165 mole) previously dissolved in water (3 cc.). The mixture was refluxed for two hours, diluted with water, made alkaline with potassium carbonate, and refluxed for a further half hour. The resulting solution was cooled and extracted with chloroform. The extract after drying and evaporation yielded an oil which almost all distilled at 135-140° at 0.2 mm., n_D^{20} 1.6031, wt. 1.37 gm. The distilled ketone was dissolved in petroleum ether (b.p. 50-60°) and it crystallized when the saturated solution was allowed to evaporate at room temperature. It consisted of colorless stout prisms, m.p. 54-55°. Calculated for $C_{11}H_8ON_2$: C, 71.73; H, 4.38; N, 15.21%. Found: C, 71.36; H, 4.30; N, 15.12%. This ketone has been prepared directly by Wibaut and his co-workers (4, 16).

The monopicrate was obtained by mixing alcoholic solutions of 2,2′-dipyridylketone and picric acid. It consisted of golden yellow prismatic needles, m.p. 180-1°, becoming deep orange at about 120°. The literature records m.p. 181-2° (4). Calculated for $C_{11}H_8ON_2 \cdot C_6H_3O_7N_3$: C, 49.40; H, 2.68; N, 16.95%. Found: C, 49.48; H, 2.52; N, 17.16%.

2,2′-Dipyridylketone semicarbazone was obtained by a method similar to that reported in the literature (4). It separated from ethanol as colorless plates, m.p. 220-221°. The recorded melting point is 218-9° (4). Calculated for $C_{12}H_{11}ON_5$: C, 59.74; H, 4.60; N, 29.03%. Found: C, 60.05; H, 4.73; N, 28.84%.

2,2′-Dipyridylketoxime

The ketone (0.74 gm., 0.04 mole) was dissolved in a mixture of ethanol (7 cc.) and water (1.5 cc.) and to the solution were added hydroxylamine hydrochloride (0.45 gm., 0.065 mole) and powdered sodium hydroxide (0.7 gm.). The mixture was refluxed for 5 min., allowed to cool, diluted with water, and neutralized with dilute hydrochloric acid. On standing long pale pink needles of the oxime separated (0.801 gm.). Crystallization from aqueous methanol gave the oxime as fine colorless needles, m.p. 141-142.5°. Calculated for $C_{11}H_9ON_3$: C, 66.17; H, 4.54; N, 21.05%. Found: C, 65.78; H, 4.53; N, 20.96%.

Beckmann Rearrangement of the Oxime

The finely powdered oxime (0.50 gm.) was added to phosphorus pentachloride (1.0 gm.) suspended in dry ether (100 cc.). The mixture was refluxed for 15 min., during which the color changed from yellow to brown. The ether solution was added to hot water, neutralized with potassium carbonate, and evaporated to a small bulk. On cooling fine needles separated (0.2 gm.) which after recrystallization from hot water gave hairlike needles of 2-pyridyl-2′-picolinamide melting at 117-8° either alone or in admixture with an authentic specimen. Calculated for $C_{11}H_9ON_3$: C, 66.17; H, 4.54; N, 21.05%. Found: C, 65.92; H, 4.45; N, 20.70%.

2-Pyridyl-2'-picolinamide

2-Picolinic acid chloride was prepared by the method of Späth and Spitzer (15). The chloride (0.2 gm.) was added to 2-aminopyridine (0.14 gm.) in benzene (10 cc.). The mixture which became dark and evolved heat was allowed to stand for one-half hour and poured into water. Sodium carbonate was added to neutralize the solution which was boiled to remove the benzene. The aqueous solution left was decolorized with charcoal and the filtrate allowed to cool when it deposited the amide as fine almost colorless needles. After recrystallization from hot water it consisted of colorless hairlike needles, m.p. 117-8°.

β-Bromomethylmalonic Ester

This ester was prepared by the method of Simonsen (14). It was a colorless liquid with an acrylic acid smell, b.p. 89-91° at 1.0 mm., n_D^{20} 1.4468. Calculated for $C_8H_{13}O_4Br$: C, 37.97; H, 5.18; Br, 31.58%. Found: C, 40.41; H, 5.54; Br, 27.58%. This product contained about 85% of the bromo compound, the main impurity probably being ethyl methylenemalonate; it was used without further purification.

Condensation of 2,2'-Dipyridylmethane with Ethyl Bromide

Bromobenzene (8.6 gm., 0.055 mole) in ether (10 cc.) was added with stirring to lithium chips (0.73 gm., 0.051 mole) suspended in ether (100 cc.) in a nitrogen atmosphere, and stirring was maintained for two hours. When all the lithium had dissolved the solution was cooled to 0° and dipyridylmethane (6.0 gm., 0.035 mole) in ether (10 cc.) was added slowly. Orange crystals of the lithium salt separated. After stirring for 30 min. freshly distilled ethyl bromide (5.45 gm., 0.055 mole) in ether (20 cc.) was added. The mixture was stirred at room temperature for six hours in the course of which the color changed from orange to pale yellow. Water was then added and the ether layer separated and extracted with dilute hydrochloric acid. The acid liquor was alkalized with potassium carbonate and extracted with ether. The ether extract was dried, evaporated on the steam bath, and the residual oil (6.23 gm.) was distilled *in vacuo* to give 5.9 gm. of αα'-(2-dipyridyl)-propane, b.p. 125° at 0.2 mm., n_D^{20} 1.5631. Calculated for $C_{13}H_{14}N_2$: C, 78.78; H, 7.12; N, 14.13%. Found: C, 79.16; H, 7.26; N, 14.02%. On mixing alcoholic solutions of the base with two equivalents of picric acid an immediate separation of the picrate occurred. Recrystallization from aqueous ethanol gave lemon yellow monoclinic prisms of the dipicrate of αα'-(2-dipyridyl)-propane, m.p. 170.5-171°. Calculated for $C_{13}H_{14}N_2 \cdot 2C_6H_3O_7N_3$: C, 45.74; H, 3.07; N, 17.07%. Found: C, 45.65; H, 3.07; N, 16.74%.

Condensation of Dipyridylmethane with β-Bromomethylmalonic Ester

*Lithio*dipyridylmethane was obtained as described above from lithium chips (1.38 gm., 0.2 gm. atoms), bromobenzene (15.7 gm., 0.1 mole), and dipyridylmethane (12.75 gm., 0.075 mole) in ether (200 cc.). The orange suspension of the lithium salt was cooled to 0° and β-bromomethylmalonic ester (29.8 gm.-0.1 mole, assuming that the content of bromo compound is 85%) in ether (50 cc.) was added slowly. The orange color slowly disappeared. After all the bromo compound had been added, the mixture was stirred for one hour. Water was then added, followed by hydrochloric acid until the aqueous layer was slightly acidic.

The aqueous layer was separated, neutralized with potassium carbonate, and extracted with chloroform. The dried extract was distilled on the steam bath; it left an oil (20.37 gm.) which was distilled *in vacuo* up to 140° at 0.1 mm. A colorless oil distilled (6.73 gm., n_D^{20} 1.5765) which consisted mainly of unchanged dipyridylmethane. The undistilled orange residue was used in the next stage of the synthesis without further purification. A small quantity of this residue was nevertheless distilled in a high vacuum, but the process was accompanied by considerable decomposition. The distillate thus obtained of the diester of αα-(2-dipyridyl)-propane-γγ-dicarboxylic acid was an orange viscous oil, b.p.180-200° at 0.05 mm., n_D^{20} 1.5245. Calculated for $C_{19}H_{22}O_4N_2$: C, 66.65; H, 6.48; N,8.18%. Found: C, 65.81; H, 6.42; N, 8.38%. The diester was only weakly basic, potentiometric titration showing that the pK value in 50% methanol of the more basic nitrogen was only 3.65. Attempts to prepare a crystalline picrate failed.

Reduction of the Diester

The diester (4.0 gm.) dissolved in glacial acetic acid (50 cc.) was hydrogenated over Adams' catalyst (1.0 gm.) at a pressure of 50 lb. per sq. in. for 20 hr. The mixture was filtered, the filtrate diluted with water, and neutralized with potassium carbonate. A colorless gum separated which was extracted with chloroform. The extract after drying and evaporation yielded a pale yellow viscous oil (4.0 gm.) which was strongly basic. The oil was heated at 130° at 1 mm. for 20 hr. and the volatile products that were evolved were condensed in a trap cooled with liquid air. The contents of the trap were treated with α-naphthyl isocyanate and the resulting white precipitate was dissolved in petroleum ether. The solution was filtered and concentrated; on cooling it deposited α-naphthyl ethyl carbamate as colorless needles, m.p. 79-80° either alone or in admixture with an authentic specimen. The oil lost 0.8 gm. during the heating *in vacuo*; there was left a brown residue which distilled at 200° at 0.001 mm., giving 1.26 gm. of colorless oily crystals. These were dissolved in benzene (50 cc.) and the solution diluted with an equal volume of petroleum ether (b.p. 50-80°). A quantity of crystals separated (0.35 gm.), m.p. 250-260°, which were sublimed at 200° at 0.001 mm. The sublimate was crystallized from ethanol – petroleum ether from which the dilactam *A* separated as glistening white plates, m.p. 262-263°. Calculated for $C_{15}H_{22}O_2N_2$: C, 68.67; H, 8.45; N, 10.68%. Found: C, 67.93; H, 8.47; N, 10.89%. The dilactam *A* was neutral (no inflection on potentiometric titration curve) and attempts to prepare a picrate failed. It was sparingly soluble in ether but readily soluble in ethanol.

The mother liquor from the dilactam *A* was chromatographed on alumina (activity 1 – 2). The fractions eluted with benzene consisted of orange yellow viscous oils probably consisting of partially ring-closed lactams. The fraction eluted with benzene containing 10% methanol, when evaporated, yielded colorless crystals, m.p. 150-158°, which were sublimed at 150° at 0.001 mm. The white sublimate of the dilactam *B* weighed 0.25 gm. and melted at 161-2°. Calculated for $C_{15}H_{22}O_2N_2$: C, 68.67; H, 8.45; N, 10.68%. Found: C, 68.55; H, 8.54; N, 10.54%. Like its isomer, Dilactam *B* was neutral and attempts to make salts were not successful.

Reduction of Dilactam A. ψ-Sparteine A

The dilactam *A* (0.200 gm.) was dissolved in pure sodium-dried dioxan (20 cc.) and to the cooled solution was added a 4% solution of lithium aluminum hydride in ether (2.5 cc.). The reaction mixture was refluxed in a dry nitrogen atmosphere for four hours, allowed to cool, and then cautiously diluted with methanol containing a little water. The precipitated aluminum hydroxide was filtered, washed with hot ethanol, and the combined filtrate and washings evaporated to dryness *in vacuo*. There remained a syrupy residue which was diluted with water and extracted with ether. The extract was dried and distilled on the steam bath to remove the solvent. A pale yellow oil remained (0.153 gm; theory 0.178 gm.) which was distilled *in vacuo*. A colorless oil was thus obtained, b.p. 80° at 0.2 mm., n_D^{20} 1.5308. The oil became yellow and tarry on standing in air. Calculated for $C_{15}H_{26}N_2$: C, 76.86; H, 11.18; N, 11.96%. Found: C, 76.94; H, 11.43; N, 11.79%. Potentiometric titration in 50% methanol indicated a molecular weight of 238 ($C_{15}H_{26}N_2$ requires 234), and the pK values for the two nitrogens, 11.0 and 4.0 (sparteine in 50% methanol has pK values of 11.3 and 4.0).

Reduction of the Dilactam B. ψ-Sparteine B

The dilactam *B* (0.200 gm.) was reduced in dioxan with a 4% ether solution of lithium aluminum hydride exactly as Dilactam *A* and the product isolated in the same way. The yield of ψ-sparteine *B* prior to distillation was 0.176 gm. The base boiled at 80-90° at 0.2 mm. and was obtained as a colorless oil, n_D^{20} 1.5295 which became yellow and tarry on standing in contact with air. Calculated for $C_{15}H_{26}N_2$: C, 76.86; H, 11.18; N, 11.96%. Found: C, 76.95; H, 11.15; N, 12.14%. The pK values obtained by potentiometric titration in 50% methanol were 11.0 and 4.5.

Attempted Resolution of ψ-Sparteine A

ψ-Sparteine *A* (0.25 gm.) was dissolved in ethanol and *d*-β-camphorsulphonic acid (0.50 gm.) was added to the solution which was then concentrated. The salt which separated was recrystallized from acetone from which it was deposited as fine colorless needles, m.p. 246-8° with darkening, $[\alpha]_D^{25} + 31.0 \pm 0.2°$ ($c = 1.51$ in absolute ethanol). Calculated for $C_{15}H_{26}N_2(C_{10}H_{16}O_4S)_2$: C, 60.14; H, 8.36; N, 4.01%. Found: C, 59.44; H, 8.28; N, 3.56%. Repeated recrystallization did not change the specific rotation. The free base recovered from the salt had no rotation in ethanol.

ψ-Sparteine *A* was also treated with *l*-β-camphorsulphonic acid. ψ-Sparteine *A*-*l*-β-camphorsulphonate crystallized from acetone as colorless needles, m.p. 246-8°. $[\alpha]_D^{25} - 31.6 \pm 0.2°$ ($c = 1.45$ in absolute ethanol). Repeated crystallization did not change the rotation.

ψ-Sparteine A Monopicrate

On adding a solution of the base (50 mgm.) in ethanol to a solution of picric acid (50 mgm.) in the same solvent fine prisms separated. Crystallization from ethanol gave fine microscopic prisms of the monopicrate, m.p. 124°. Calculated for $C_{15}H_{26}N_2 \cdot C_6H_3O_7N_3$: N, 15.11%. Found: 15.24%.

ψ-Sparteine A Diperchlorate

A quantity of the base was dissolved in ethanol and the solution made acid to congo red by the dropwise addition of 72% perchloric acid. The perchlorate which separated almost immediately was crystallized from 95% ethanol. It consisted of colorless prisms containing ethanol of crystallization. After drying at 110° it melted at 246-8°. Calculated for $C_{15}H_{26}N_2 \cdot 2HClO_4$: C, 41.38; H, 6.48; N, 6.43%. Found: C, 41.59; H, 6.52; N, 6.08%.

Attempted Resolution of ψ-Sparteine B

When the freshly distilled ψ-sparteine *B* was treated with *d*- or *l*-β-camphorsulphonic acid in ethanol no crystalline product was obtained.

When the base (0.18 gm.) was treated with *d*-6,6′-dinitro-2,2′-diphenic acid (0.26 gm.) in ethanol, a salt crystallized out. Crystallization from ethanol, in which it was sparingly soluble, yielded the salt as pale yellow prismatic needles, m.p. 235-6°, $[\alpha]_D^{26} + 162 \pm 2°$ ($c = 0.153$ in absolute ethanol). Calculated for $C_{15}H_{26}N_2 \cdot C_{14}H_8O_8N_2$: C, 61.47; H, 6.05; N, 9.89%. Found: C, 61.62; H, 5.93; N, 9.77%. The base recovered from this salt showed no rotation in ethanol.

ψ-Sparteine B Monopicrate

This salt was obtained by treating an ethanolic solution of the base with an equivalent quantity of picric acid. On crystallization from ethanol the monopicrate was obtained as stout yellow prisms, m.p. 165-6°. Calculated for $C_{15}H_{26}N_2 \cdot C_6H_3O_7N_3$: C, 54.42; H, 6.30; N, 15.11%. Found: C, 54.54; H, 5.98; N, 15.00%.

ψ-Sparteine B Diperchlorate

The base in ethanol was treated with 72% perchloric acid in the usual way. The perchlorate separated on the addition of ether and after recrystallization from ethanol–ether was obtained as small microscopic prisms, m.p. 232-3°. Calculated for $C_{15}H_{26}N_2 \cdot 2HClO_4$: C, 41.38; H, 6.48; N, 6.43%. Found: C, 41.85; H, 6.37; N, 6.36%.

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X-RAY DIFFRACTION PATTERNS OF DINITROPHENYL DERIVATIVES OF AMINO COMPOUNDS[1]

By H. M. Rice and F. J. Sowden

ABSTRACT

The 2,4-dinitrophenyl derivatives of a number of amino compounds have been prepared and their X-ray powder diffraction measurements made. This appears to be the first time this information has been obtained and it is submitted as a method of identification.

INTRODUCTION

In recent years the reaction of 1-fluoro-2,4-dinitrobenzene with proteins and protein derivatives has been used a great deal in work on the structure of proteins and in similar problems (2, 6, 7, 8, 9, 10, 11). The free amino groups react with the reagent to form dinitrophenyl derivatives and identification of the derivatives formed shows which amino groups were free in the intact protein. X-ray powder diffraction patterns are being used to an increasing degree in the identification of organic compounds (3, 5) and are very useful since a high degree of purity is not required. The 2,4-dinitrophenyl derivatives of a number of amino acids were prepared by the methods described by Abderhalden and Blumberg (1), Sanger (8), and Porter and Sanger (7) as standards of reference for work on the free amino groups of soil organic matter and their X-ray powder diffraction patterns determined.

X-RAY EXAMINATION

Diffraction patterns of the 2,4-dinitrophenyl derivatives of the amino acids were obtained with a Philips Norelco X-ray spectrometer (1950 model). The samples were ground to pass a 350-mesh sieve and placed in spectrometer frames. Considerable difficulty was encountered in sieving the samples and in preparing nonoriented material for the spectrometer examination.

Preliminary investigation showed that Cu $K\alpha$ radiation was too strong for satisfactory spectrometer patterns. Consequently Fe $K\alpha$ radiation ($\lambda = 1.93597$ Å) with manganese filter at 45 kv., 20 ma., and a rate meter setting of 2-06-8 was used. While this was satisfactory for the most prominent lines, it was felt that powder photographs would be better for the weaker lines. Powder samples therefore were made with the material that had been prepared for the spectrometer, using fine glass tubes of 0.2 mm. bore. Philips 114.5 mm. diameter cameras were used with an exposure time of 18 hr.

RESULTS AND DISCUSSION

The analyses of the dinitrophenyl derivatives are reported in Table I. The determinations for carbon, hydrogen, and nitrogen were made in the microanalytical laboratory of Dr. R. Dietrich, Zurich, Switzerland. The nitrogen content was also measured in this laboratory by the Friedrich-Kjeldahl method.

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TABLE I

COMPOSITION AND MELTING POINTS OF DINITROPHENYL DERIVATIVES

Compound	Composition (%)						Melting point (°C.)	
	Found			Calculated			Found	Reported
	C	H	N	C	H	N		
2,4-dinitroaniline	39.5	2.68	22.8	39.3	2.70	22.9	179	176 (188) (4)[2]
dnp[1]-glycine	39.7	2.82	17.4	39.8	2.90	17.4	206	205 (1)
dnp-*dl*-α-alanine	41.2	3.61	16.2	42.3	3.53	16.5	178	178 (1)
dnp-β-alanine	42.5	3.52	16.6	42.3	3.53	16.5	121 – 5	—
dnp-*dl*-valine	46.5	4.56	14.8	46.6	4.60	14.8	185	185 (1)
dnp-*dl*-serine	40.1	3.40	15.6	39.9	3.30	15.5	200	199 (7)
dnp-*dl*-leucine	48.6	5.19	14.1	48.5	5.10	14.1	126	203 (1)
dnp-*dl*-aspartic acid	40.3	3.16	14.1	40.2	3.00	14.0	190	196 (7)
ε-dnp-*l*-lysine.HCl.H_2O	38.9	5.37	15.4	39.5	5.20	15.4	189	186 (8)
bis-dnp-lysine	45.1	3.68	17.4	45.1	3.77	17.6	174	146 (7)
dnp-*l*-histidine	44.5	2.85	20.0	44.4	2.67	20.1	228	250 (1)
dnp-*l*-asparagine	39.9	3.35	18.7	40.2	3.40	18.8	185	191 (1)

[1]*dnp—dinitrophenyl.*
[2]*Small numbers in brackets indicate literature references.*

PLATE I

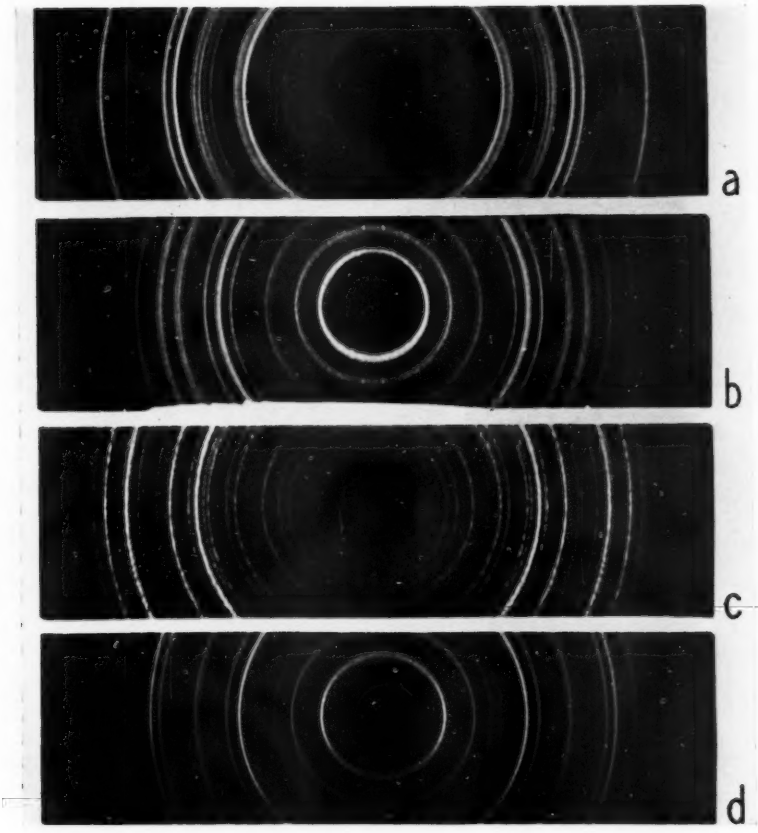

FIG. 1. Typical powder diagrams of dinitrophenyl derivatives with Fe $K\alpha$ radiation.
a. dnp-*dl*-aspartic acid.
b. dnp-*dl*-leucine.
c. dnp-*dl*-serine.
d. dnp-*dl*-valine.

TABLE II

DIFFRACTION DATA*

1. 2,4-dinitro-aniline		2. dnp-glycine		3. dnp-*dl*-α-alanine		4. dnp-β-alanine	
d(Å)	I/I_0	*d*(Å)	I/I_0	*d*(Å)	I/I_0	*d*(Å)	I/I_0
7.78	1	9.68	3	10.4	7	15.1	3
7.00	1	7.39	3	9.83	10(1)	9.79	2
6.40	7	6.36	1	7.99	2	7.60	4
6.26	3	5.91	4	6.63	1	7.24	2
5.72	1	5.11	1	5.99	2	6.37	2
5.24	1	4.73	1	5.39	2	5.85	3
4.79	8(3)	4.57	9	5.14	4	5.41	1
4.55	8(2)	4.36	3	4.98	1	5.05	3
4.32	6	4.21	1	4.66	9(2)	4.92	4
3.83	6	4.10	2	4.46	1	4.70	2
3.75	1	3.98	5	4.22	3	4.54	8(2)
3.58	2	3.75	10(1)	4.10	5	4.29	4
3.40	2	3.66	10(2)	3.92	1	4.21	3
3.32	1	3.52	3	3.75	1	4.12	6
3.20	10(1)	3.26	4	3.67	8(3)	3.87	10(1)
3.10	7	3.18	4	3.46	1	3.77	4
2.98	1	3.12	2	3.33	1	3.59	3
2.94	2	3.08	10(3)	3.29	7	3.51	5
2.86	4	2.93	1	3.20	1	3.39	7(3)
2.74	2	2.84	1	3.12	7	3.27	5
2.68	2	2.70	3	3.06	1	3.23	7(3)
2.63	1	2.62	1	2.97	1	3.16	1
2.56	1	2.55	1	2.90	6	3.01	7(3)
2.51	4	2.51	1	2.75	1	2.93	1
2.46	1	2.44	1	2.64	2	2.76	4
2.44	2	2.40	2	2.58	1	2.64	1
2.39	1	2.28	4*d*	2.50	1	2.54	1
2.33	1	2.18	1	2.41	1	2.44	1*d*
2.28	2	2.15	1	2.32	3	2.34	2
2.24	1	2.13	2	2.05	1	2.27	1
2.20	2	2.09	1	1.97	1	2.24	1
2.15	1	2.06	2	1.72	1	2.20	1
2.08	1*d*	2.03	1			2.15	1*d*
2.06	1	2.00	2			2.03	2
1.94	4	1.94	3			1.99	1
1.91	1	1.92	1			1.94	1
1.88	2	1.87	1			1.92	1
1.83	1	1.83	1			1.85	1
1.79	2	1.81	1			1.80	1
1.75	1	1.79	1			1.63	1
1.72	1	1.75	2				
1.69	2	1.60	1				
1.58	2	1.37	1				
1.54	1						
1.50	1						

*d(Å) = *interplanar spacings in Ångstroms*; I/I_0 = *estimated relative intensity*; (1) = *strongest line*; (2) = *second strongest line*; (3) = *third strongest line*; *d* = *diffuse*; *dnp* = *dinitrophenyl.*

TABLE II (*Continued*)

5. dnp-*dl*-valine		6. dnp-*dl*-serine		7. dnp-*dl*-leucine		8. dnp-*dl*-aspartic acid	
d(Å)	I/I_0	*d*(Å)	I/I_0	*d*(Å)	I/I_0	*d*(Å)	I/I_0
12.0	9(2)	9.42	3	13.9	10(1)	8.34	2
8.09	5	8.38	4	10.0	2	6.59	1
6.85	3	7.10	5	9.58	6	5.75	10(1)
6.19	1	6.17	2	7.18	1	5.55	2
5.96	1	5.61	5	6.91	7(2)	5.38	5
5.48	1	5.28	4	5.23	6	5.34	1
5.17	10(1)	4.92	3	5.03	1	5.05	2
4.97	1	4.68	5	4.87	7(3)	4.83	3
4.75	7	4.56	5	4.75	5	4.57	4
4.47	2	4.36	10(1)	4.43	6	4.44	3
4.34	1	4.18	5	4.32	1	4.27	5
4.18	1	3.87	1	4.18	1	4.16	5
4.06	2	3.78	6(3)	4.04	2	4.08	3
3.96	5	3.55	3	3.95	2	3.93	1
3.87	2	3.41	3	3.85	3	3.75	9½(2)
3.80	1	3.34	3	3.77	3	3.66	1
3.65	1	3.27	4	3.67	1	3.57	9(3)
3.54	1	3.11	8(2)	3.52	5	3.47	1
3.44	1	3.07	2	3.46	2	3.38	2
3.35	6	3.00	2	3.34	2	3.30	1*d*
3.21	9(3)	2.94	2	3.23	1	3.15	1
3.15	2	2.88	5	3.18	3	3.09	1
2.99	4*d*	2.83	1	3.10	2	3.04	2*d*
2.84	1	2.76	4	3.04	2	2.93	1
2.76	1	2.73	3	2.91	1	2.87	2
2.71	1	2.64	1	2.88	1	2.76	7
2.66	2	2.62	1	2.77	1	2.68	2
2.51	1	2.52	3	2.68	2	2.60	2
2.47	2	2.45	3	2.62	1	2.56	2
2.33	1	2.39	2	2.53	1	2.54	1
2.23	1	2.34	2	2.47	2	2.44	2
2.19	1	2.31	2	2.41	1	2.28	2
2.10	2	2.27	1	2.33	1	2.15	1
1.92	1	2.23	1	2.27	1	1.99	2
1.86	1	2.17	2	2.15	1	1.93	1
		2.13	2	2.11	1	1.90	1
		2.10	3	2.00	1	1.87	1
		2.07	2	1.96	1	1.84	1
		2.04	1	1.91	1	1.79	1
		2.02	1	1.87	1	1.74	1
		1.99	2	1.84	1	1.62	1
		1.94	4	1.39	1	1.60	1
		1.87	2*d*	1.15	1*d*		
		1.80	1	1.09	1*d*		
		1.75	1				
		1.64	1				
		1.57	1				
		1.56	1				
		1.53	1				
		1.50	1				
		1.45	1				
		1.18	1				
		1.17	1				

TABLE II (*Concluded*)

9. ε-dnp-*l*-lysine.HCl.H_2O		10. *bis*-dnp-lysine		11. dnp-*l*-histidine		12. dnp-*l*-asparagine	
d(Å)	I/I_0	*d*(Å)	I/I_0	*d*(Å)	I/I_0	*d*(Å)	I/I_0
17.5	5	13.5	2	16.6	1	10.9	2
9.50	10(1)	11.1	5	15.5	4	7.70	7
8.75	1	8.55	3	7.72	1	5.49	5
7.76	1	7.72	1	7.55	3	5.06	4
6.65	3	6.32	4	7.09	7	4.85	7
6.28	5	6.05	2	6.24	8	4.62	8(3)
5.01	1	5.55	10(1)	5.49 } Band	3	4.20	3
4.87	2	5.20	1	5.36 }		3.91	10(1)
4.72	3	4.90	2	5.19	1	3.85	1
4.39	4	4.66	8	5.01	1	3.72	1
4.25	1	4.38	5	4.83	6	3.46	2
4.13	10(2)	4.29	1	4.62	3	3.41	1
3.98	1	4.06	5	4.37	7	3.33	10(2)
3.85	10(3)	3.72	2	4.24	1	3.16	8(3)
3.68	2	3.61	9(2)	4.08	1	3.00	1
3.53	3	3.47	8(3)	4.00	1	2.97	2
3.41	2	3.29	2	3.88	9(2)	2.84	1
3.28	1	3.13	2	3.75	4	2.75	4
3.17	5	3.01	1	3.61	1	2.65	1
3.12	8	2.91	1	3.50	10(1)	2.58	1
2.99	3	2.83	1	3.32	4	2.54	1
2.92	1	2.60	2	3.22	1	2.46	1
2.86	1			3.15	1	2.41	2
2.81	1			3.05	1	2.35	1
2.74	1			2.95	8(3)	2.30	2
2.61	3			2.74	2	2.24	1
2.53 } Band	1			2.37	1	2.18	3
2.47 }				2.23	1	2.14	1
2.35	2			2.17	1	2.12	1
2.30	1			2.10	2	2.08	1
2.25	1			2.00	1	2.00	1
2.21	1			1.39	2*d*	1.94	1
2.14	1			1.15	4*d*	1.84	1
2.10	1			1.09	2*d*		
2.06	3						
2.00	1						
1.93	1						
1.89	1						
1.88	1						
1.83	1						
1.74	1						
1.69	1						
1.57	1						

Most of the compounds melted with some decomposition and, in a few instances, the melting points differed considerably from those recorded in the literature. However, the compounds seemed to be pure when tested chromatographically and a second preparation and purification gave compounds melting at the same temperature.

The results of X-ray analysis are given in Table II and four typical powder diagrams are shown in Fig. 1. The I/I_0 values were estimated visually from 1 to 10 and the three strongest lines are indicated as 1, 2, and 3.

It can be seen from Table II and Fig. 1 that all the compounds examined gave distinct powder patterns; even with such similar compounds as α- and β-alanine, the patterns were quite distinct. In work on protein structure, frequently only small samples of somewhat impure material are available. In such cases, X-ray powder patterns are very useful for purposes of identification.

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AN ULTRAMICRO METHOD FOR QUANTITATIVE DETERMINATION OF AMINO ACIDS[1]

By Eunice F. Wellington[2]

ABSTRACT

A simple, quantitative method for determination of amino acids in about 300 μgm. protein is described. The amino acids are separated by two-dimensional paper chromatography. The chromatograms are sprayed with 2% ninhydrin and the color is developed under controlled and standardized conditions. The colored areas are cut out, eluted, and the color intensity is read in a photometer. The following factors have been shown to affect the color reaction of ninhydrin with amino acids on paper chromatograms: the quality of the ninhydrin; its concentration; time of color development; and the environmental temperature and humidity. Complete oxidation of the hydrolyzate is necessary for accurate quantitative determinations of cysteine, cystine, glutamic acid, aspartic acid, methionine, and valine. Strict standardization of the length of the solvent run is also important for reproducibility in the analyses of some amino acids.

Bovine serum albumin has been analyzed by this method and the results found to be in excellent agreement with previously published values.

INTRODUCTION

Work on insect viruses in progress in this laboratory required a simple method for the ultramicro determination of amino acids in complex mixtures. The present investigation was undertaken to fulfill this need.

Microbiological techniques (28) and chromatography on starch (24) or on polystyrene resin columns (25) have been applied successfully to the problem of quantitative analyses of amino acids. However, these methods require more material than is usually available from insect virus stocks and, in addition, require elaborate equipment or considerable labor.

Since the introduction of paper chromatography for the separation and identification of amino acids in microgram quantities (12), many attempts have been made to adapt this technique to quantitative work. Some of the recently published approaches have been: (*a*) estimation of the ninhydrin color directly on paper chromatograms, (*b*) preliminary elution of amino acids from the chromatogram followed by estimation of the ninhydrin color, and (*c*) estimation of the soluble copper salts of the amino acids (22, 37). Application of the ninhydrin reaction seemed the most promising approach.

The majority of the published methods for ninhydrin reaction on paper involve a large replication of samples and simultaneously prepared standards (1–5, 11, 15, 16, 26, 27), as results are reported to vary from day to day. The matter is further complicated because most of these methods are applicable to one-dimensional chromatography only.

Moore and Stein (23) investigated the reaction of ninhydrin with amino acids in their work with starch columns, and developed a modified reagent containing a reducing agent, which gives easily reproducible results under

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their conditions but has the disadvantage of giving an equimolar response with ammonia.

Boissonnas (8) and Fowden (17) claimed good results in determinations of amino acids eluted from paper chromatograms by the use of slight variations of this reagent. With either method, it is difficult to locate some of the amino acids. To establish the positions of amino acids, Fowden relied on their fluorescence on filter paper after heating. This is not a reliable method since amino acids do not fluoresce on all papers nor do they all fluoresce in low concentrations (21). Boissonnas (7) devised an ingenious "hedgehog" for spotting the paper with ninhydrin as a means of locating amino acids. Well-separated amino acids are easily discerned with this apparatus, but it is difficult to ascertain the boundaries of those lying close together.

Both Boissonnas and Fowden recognize the necessity of treating the paper with sodium hydroxide to overcome the variability due to ammonia in the blank. As a further precaution, Boissonnas used ammonia-free solvents for separating the amino acids (6). However, "tailing" occurred with the chemicals available in this laboratory and, therefore, the solvent pairs suggested by Boissonnas did not give adequate separation. Chromatographic solvents that gave adequate separation (35) gave high and variable paper blanks with Boissonnas' reagent, thus invalidating the results.

Recently, Thompson *et al.* (32, 33) described another method for quantitative two-dimensional paper chromatography. This method requires special washing of the papers before use and special tanks for developing the ninhydrin color in an atmosphere of carbon dioxide and ethyl alcohol. In addition, about 2 mgm. protein are required for one analysis. The method described below is much simpler, requires less time, and one complete analysis can be carried out with about one-seventh the amount of protein, i.e., 300 μgm.

EXPERIMENTAL AND RESULTS

Factors Affecting the Reproducibility of the Ninhydrin Reaction on Paper

Quality of Chemicals

Amino acids.—All amino acids should be checked chromatographically for purity in at least two solvents before use.

Phenol.—Merck reagent grade phenol must be distilled by the method of Draper and Pollard (14) before use. This gives an almost white background color to the papers that provides low blanks of 0.004–0.016 optical density at 570 mμ. Furthermore, some batches of Whatman No. 1 filter paper ("For Chromatography") only give adequate separation of histidine, lysine, and arginine when distilled phenol is used.

Ninhydrin.—This was obtained from Dougherty Chemicals (87–34 134th Street, Richmond Hill, New York). A variation in the intensity of the colored ninhydrin reaction product was noted with different batches of ninhydrin. This variation was negligible for some amino acids, but considerable for

others. Therefore, it is necessary to check the color yields for all the amino acids when a new lot of ninhydrin is used.

Quality and Size of Paper

Whatman No. 1 and No. 4 papers are suitable for use in the method described below. Schleicher and Schüll paper No. 507 is not suitable, since the colored ninhydrin product is not completely eluted with a propanol–water solution.

There has been some controversy over the effects of varying the dimensions of chromatograms. Woiwod (37) has reported a loss of glycine, and Thompson and Steward (32) reported loss of all amino acids when dimensions varied. On the other hand, Fowden and Penney (18) found no change in amino acids correlated with length of solvent runs. The present tests demonstrated that the initial dimensions chosen for a chromatogram should be adhered to at all times, because variability in the distances the solvents travel seriously affects the color yields of cysteic acid, glycine, threonine, lysine, arginine, and valine. On the other hand, as long as a standard size is used, losses on the paper do not affect the reproducibility and, consequently, the validity of the results.

Temperature and Humidity

The chromatogram must be dried at room temperature after each run, since Fowden and Penney (18) have shown that heating chromatograms wet with solvent causes considerable changes in or losses of amino acids. After the second run the papers are kept at room temperature for 24 hr. and then sprayed with the ninhydrin solution. The papers must be rapidly dried (by air movement) immediately after the spraying.

Earlier workers with one-dimensional chromatography found it necessary to run large series of standard amino acids with every unknown because of day-to-day variability of color yields. This variability was assumed to be due to changing temperature and humidity, although no definite tests of this assumption were made. Recently, Thompson *et al.* (33) showed that a constant temperature was necessary for reproducible results, and they took further precautions by developing color in special tanks containing an atmosphere of carbon dioxide and ethyl alcohol.

Special atmospheres to eliminate water vapor are unnecessary. It is true that color intensities of standards vary if color development takes place at different temperatures and humidities. In fact, the situation in an uncontrolled environment is more complex than is generally realized, because the color yields from different amino acids are affected differently by changes in environmental temperature and humidity. This means that an amino acid marker added to two-dimensional chromatograms is not a valid indicator of the changes that occur in all other acids in the chromatograms.

Since moist air inhibits the development of color even when the papers are transferred later to a dry atmosphere, suitable and convenient conditions are $20 \pm 1°$ C. and $40 \pm 3\%$ relative humidity. Under these conditions, the color yield follows Beer's Law.

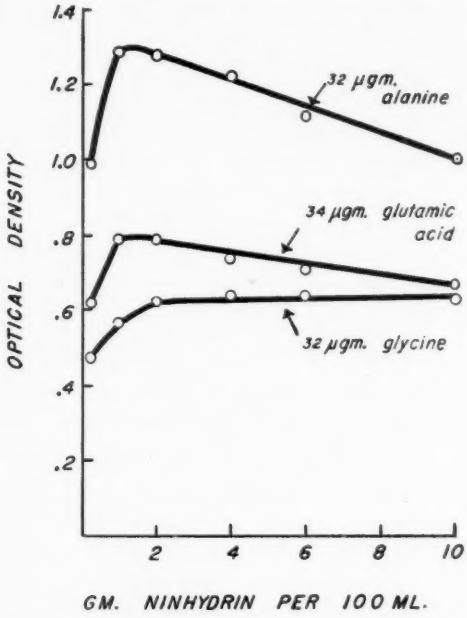

FIG. 1. The effect of ninhydrin concentration on the colored product formed with amino acids.

Concentration of Ninhydrin

The ninhydrin is dissolved in absolute ethyl alcohol, in which amino acids are only slightly soluble. This prevents spreading of amino acid spots.

Most investigators have used low ninhydrin concentrations, up to about 0.25%. Recently, Thompson *et al.* (33) have used 1–2%, and the present work confirms their findings that concentrations higher than 0.25% give stronger colors and reproducible results even under the different conditions of color development. Fig. 1 shows the dependence of color yield on the concentration of ninhydrin. It can be seen that this dependence is not the same for all amino acids, but investigation of 20 amino acids showed that a 2% solution gives a nearly optimal reaction for all.

Time of Color Development

Early workers heated the chromatograms to develop the color product. Dent (13) stated that full color development takes place if sprayed papers are dried for 24 hr. at room temperature. The present work showed that maximum color development is reached at different times for the various amino acids. This is shown in Fig. 2. After 30 hr., the maximum color has developed for all amino acids and remains constant for at least another 24 hr. However, it is advisable to elute the spots as soon as possible after 30 hr., since the blank values increase after that period.

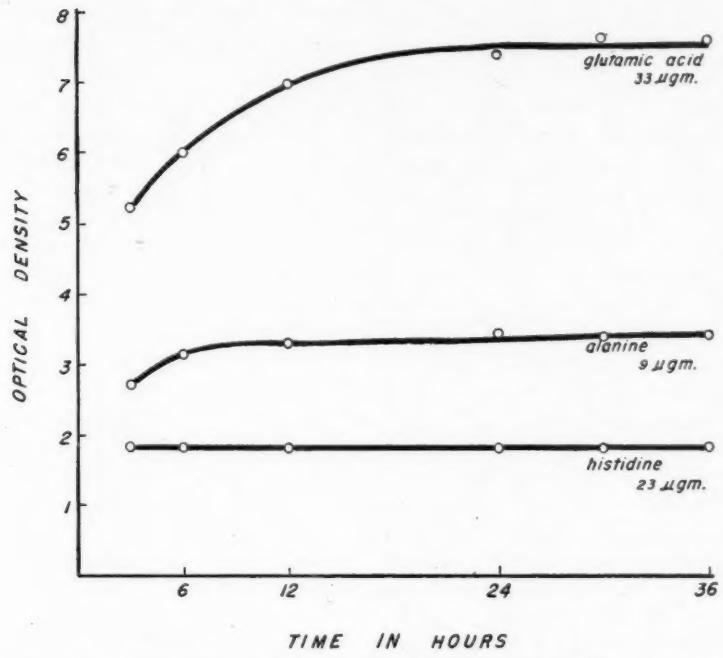

FIG. 2. The effect of time on the development of the ninhydrin colored product.

Elution and Determination of the Colored Ninhydrin Products

The colored areas are cut in strips and eluted in 50% aqueous *n*-propanol. The optical density for all amino acids except proline is read at 570 mμ. Moore and Stein (23) found that, under their conditions, the maximum optical density for the proline product was at 440 mμ. Thompson *et al.* (33) recommended 330 mμ. However, under the conditions of the method described here, the maximum absorption occurred at 350 mμ, and this wave length was used for this acid.

Oxidation of Sulphur-containing Amino Acids

Controlled oxidation of cysteine, cystine, and methionine is essential for reproducible results. This is achieved only with great difficulty on paper but easily in a test tube.

DESCRIPTION OF THE METHOD

Hydrolysis

The hydrolyses are carried out in sealed bombs with 50 times the sample weight of constant-boiling hydrochloric acid (distilled from glass) for 16 hr. in a glycerine bath controlled at 110–112° C.

Oxidation

A volume of 30% hydrogen peroxide equal to one-fifth the volume of

hydrochloric acid used is added to the cooled hydrolyzate. The sample is well mixed and allowed to stand for at least four, but not more than five, hours.

Chromatography

Small sheets (30 cm. × 28 cm.) of Whatman No. 1 or No. 4 paper are used for the two-dimensional chromatograms. The rolled papers are placed in Petri plate covers containing the solvent, and a 7-gal. cylindrical glass jar is inverted over three papers for the duration of a run. For the second run, which requires a special atmosphere, extra chemicals are placed in a 23-cm. crystallizing dish. The Petri plates containing the solvent for the run are placed on a glass rack in this dish and the 7-gal. glass jar inverted over the whole assemblage. The first run (approx. 20 hr.) is 29 cm. past the spot of application. The paper is then trimmed to 26 cm. in the first direction (1 cm. is cut from the bottom and 3 cm. from the top of the sheet), re-rolled, and run 25 cm.

Fractions (6–15 μl. representing 100–250 μgm. of protein) of the oxidized sample are taken with self-filling ultramicro pipettes for nitrogen determinations and for chromatography. To prevent the bubbling that the peroxide may cause in the mixture, the pipettes must be kept very clean and they must be filled while being held in a horizontal or slightly inverted position. The size of the spot of application is kept smaller than 4 mm. in diameter. Not more than 2 μl. of the sample should be applied to the spot at one time. The spot is dried by a blast of air after each application until the total volume required is on the paper.

The papers are run by capillary ascent (36) at 20° C. A mixture of absolute ethanol (60 vol.), tertiary butanol (20 vol.), reagent ammonia water of specific gravity 0.90 (5 vol.), and water (15 vol.) is used for the first run (35). Water-saturated phenol in an atmosphere of cyanide and ammonia is used for the second run (12) (0.1% sodium cyanide is placed in a crystallizing dish under the papers and four drops of reagent ammonia water are run down the side of the tank at the beginning of the run). These solvent systems resolve all the amino acids except leucine, isoleucine, and phenylalanine. These three may be separated if required by a one-dimensional run in water-saturated butanol – benzyl alcohol (equal volumes) in an atmosphere of cyanide (12), but for present purposes the three have been estimated together.

Drying

After each run, the papers are dried at 20° C. in a fume cabinet. Drying is continued for six hours after the first run and for 24 hr. after the final run.

Spraying and Development

The papers are sprayed with a solution of 2% ninhydrin in absolute ethyl alcohol. Compressed air and an all-glass atomizer facilitate uniform spraying. Twelve to fourteen milliliters are sprayed over the completed two-dimensional chromatograms (25.5 × 27.5 cm. in size). The papers are rapidly dried in a fume hood, then removed to a well-ventilated room kept free from other chemical work.* They are hung for 30–36 hr. in the darkened room at 20 ± 1° C. and 40 ± 3% relative humidity.

**Exposure to hydrochloric acid fumes at any time after the papers are taken from the second run until the colored ninhydrin product is eluted may seriously affect the reproducibility of the results.*

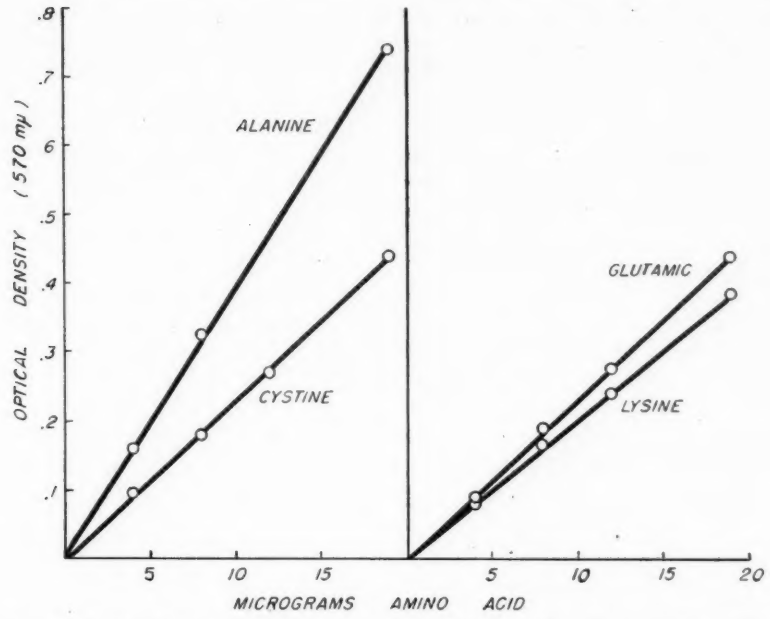

FIG. 3. Standard curves of representative amino acids after chromatography on paper by the procedure described. (Cystine was measured as cysteic acid.)

Elution and Determination

Each colored area of the paper is cut into strips and placed in a test tube containing 5 ml. of 50% aqueous *n*-propanol. Three areas are cut out for blanks: the first for proline, cut near the phenol front and equal in size to the proline spot; the second for lysine, arginine, valine, and the group leucine, isoleucine, and phenylalanine, cut just below the lysine spot and of a size equal to the largest in this group; and the third, for all the rest of the amino acids, cut above glutamic acid and of a size equal to the largest in the group. The area taken for each amino acid in each group corresponds in size to the group blank, thus eliminating the need for individual blanks. The tubes are shaken in a mechanical shaker for 10 min., then left standing for 15 min. to allow the lint from the paper to settle.

All measurements are made in a 1-cm. cell in a Beckman spectrophotometer (using a hydrogen lamp as the radiation source, and the ultraviolet-sensitive phototube) at 570 mμ, except those for proline and its blank which are read at 350 mμ. The eluted color is stable for at least six hours.

Standard Curves

Standard curves are prepared for each amino acid (Fig. 3) from data obtained from two-dimensional chromatograms carried through at the constant temperature and humidity noted above. A single series of standard curves will

Large concentrations of this acid vapor entirely prevent the development of the characteristic color on the paper.

serve for all subsequent analyses carried through under the same constant conditions. Amounts of each amino acid in the materials being analyzed are then read directly from these standard curves.

Application of the Method

An analysis of bovine plasma albumin was carried through to check the accuracy of the method. As suggested by Stein (30), a further check on the analysis was made by preparing a mixture of pure amino acids to simulate bovine serum albumin. This mixture was based on the published analyses of this protein by Stein and Moore (31). Submitting such a mixture to the conditions of hydrolysis gives a measure of the errors introduced in the later stages of hydrolysis and in the analytical technique itself. Four samples of the mixture and of the albumin (Armour lot No. 4802) were subjected simultaneously to the same conditions of hydrolysis. From each hydrolyzate, three papers were run at each of two concentrations.

An example of the agreement between replicates of three papers run at the same time is given in Table I. The results are given in terms of optical density at 570 mμ read on the Beckman spectrophotometer.

TABLE I

Reproducibility among replicates* of an albumin hydrolyzate

Amino acids	Optical density read at 570 mμ (except where noted) on the Beckman spectrophotometer		
	Paper I	Paper II	Paper III
Cysteic	.251	.253	.252
Aspartic	.126	.118	.110
Glutamic	.686	.686	.699
Serine	.204	.207	.210
Glycine	.076	.080	.069
Threonine	.196	.200	.180
Alanine	.411	.415	.442
Tyrosine oxidation product	.026	.025	.024
Methionine sulphone	.024	.025	.024
Histidine	.075	.067	.068
Lysine	.482	.482	.469
Arginine	.170	.161	.154
Valine	.255	.246	.241
Leucine + isoleucine + phenylalanine	.411	.438	.412
Proline (350 mμ)	.357	.327	.352
Blank I	.006	.006	.004
Blank II	.017	.017	.017
Blank III (350 mμ)	.166	.161	.190

**The equivalent of 175 μgm. albumin was run on each two-dimensional chromatogram.*

Table II shows the reproducibility of the results on successive hydrolyzates carried through on different days. Three papers each with the equivalent of 175 μgm. of albumin were run for every hydrolyzate. The standard errors of the means are calculated from the results of four hydrolyzates.

TABLE II

AMINO ACID COMPOSITION OF BOVINE SERUM ALBUMIN

Amino acid	Gm. amino acid residue per 100 gm. bovine serum albumin***	Standard error as per cent of the mean	Gm. amino acid per 100 gm. protein	Literature values, gm. amino acid per 100 gm. of protein
Cystine + cysteine*	5.43 ± .054	1.0	6.4	5.91 (31), 6.52 (9, 10)
Aspartic	8.79 ± .24	2.7	10.2	10.91 (31), 10.6 (9), 11.1 (19), 10.25 (29)
Glutamic	14.49 ± .12	0.8	16.5	16.50 (31), 16.9 (9), 16.6 (19), 16.95 (29)
Serine	3.70 ± .02	0.5	4.45	4.23 (31), 4.5 (9, 10), 4.9 (34)
Glycine	1.41 ± .04	2.6	1.85	1.82 (31), 1.9 (9), 1.96 (29), 2.0 (34)
Threonine	4.94 ± .14	2.9	5.90	5.83 (31), 6.5 (9, 10), 6.2 (8), 6.3 (20)
Alanine	4.75 ± .05	1.1	5.95	6.25 (31)
Tyrosine*	4.69 ± .51	11.0	5.2	5.06 (31), 5.5 (9, 29), 5.2 (20)
Methionine*	0.92 ± .01	1.1	1.05	0.92 (31), 0.81 (9, 10), 0.86 (19), 0.80 (29)
Histidine	4.03 ± .25	6.3	3.65	4.0 (31), 3.8 (9, 10), 4.1 (20)
Lysine	13.58 ± .35	2.5	12.4	12.82 (31), 12.4 (9), 12.4 (29), 12.3 (20)
Arginine	6.0 ± .16	2.7	5.85	5,90 (31), 6.2 (9, 10), 5.9 (19), 6.1 (20)
Valine	4.72 ± .13	2.8	5.55	5.92 (31), 6.5 (9), 5.4 (20), 6.6 (19)
Leucine + isoleucine + phenylalanine	18.8 ± .57	3.0	21.6	21.47 (31), 22.7 (9), 21.95 (19), 21.25 (34)
Proline**	4.63		5.5	4.75 (31), 5.6 (9), 5.1 (19), 5.5 (34)
Tryptophane	——		——	0.58 (9, 10)
Totals	100.88		112.05	

**All measured as the oxidation products.*

***Sufficient values not available as wave length for reading optical density improved for last determination.*

****Mean of four analyses on separate hydrolyzates, and its standard error.*

This table also shows results in the literature compared with those obtained by the present method. None of the author's results were corrected for losses during hydrolysis, since the standards were subjected to the same conditions as the protein. The present results are in good agreement with other published values.

DISCUSSION

The aim of this investigation—a simple, reproducible, quantitative method for analyses of micro amounts of amino acids—is achieved only when calibration curves for each amino acid are prepared under standardized chromatographic conditions. The losses that occur during travel on paper are individually compensated for each amino acid by rigid control of the length of the chromatographic run. Hence, possible differences between theoretically and experimentally obtained ninhydrin color yields are of no practical importance.

Some aspects of oxidation require further emphasis. It is generally recognized that cystine, cysteine, and methionine interfere with other amino acids on the paper, and this is usually prevented by oxidizing the amino acid mixture. In the present work, it was found that this oxidation should be carried out *in solution* instead of on the paper, in order to obtain *complete* conversion of cystine plus cysteine to cysteic acid and of methionine to methionine sulphone. Otherwise, determinations of these acids, as well as those of aspartic and glutamic acid, and valine, with which they interfere, become inaccurate. This is not generally recognized, nor is the fact that oxidation, either on paper or in solution, partially destroys tyrosine. Oxidation in solution, which is sufficient for the sulphur-containing amino acids, converts tyrosine to an oxidation product that has a different position on the paper and a low color yield, but which can still be used for determination by this method, although the error is high. This difficulty may be overcome by determining tyrosine separately from an unoxidized sample.

The results shown in Table I indicate the variation between three replicate analyses of any one hydrolyzate. If higher accuracy is required, chromatograms in triplicate are recommended. Table II shows the means and standard errors of four independent hydrolyses of bovine plasma albumin. Triplicate chromatograms of each hydrolysis were run. The analyses for cystine plus cysteine, glutamic acid, serine, alanine, and methionine were carried out with a standard error of the means of 1% or less. The errors of the means for aspartic acid, glycine, threonine, lysine and arginine, valine, and the group leucine, isoleucine, and phenylalanine were all less than 3%. The error for histidine was 6%, and that for tyrosine was 11%.

For routine work it is not necessary to prepare the calibration curves from hydrolyzed amino acid mixtures. However, if high accuracy is desired, this should be done with a mixture simulating the composition of the protein in question, as suggested by Stein (30). In this way, possible errors introduced by interaction of amino acids during hydrolysis and by the analytical technique are reduced.

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SYNTHESIS OF α-AMINO ACIDS FROM ETHYL CYANOACETATE[1]

By Paul E. Gagnon, Guy Nadeau,[2] and Raymond Côté[3]

ABSTRACT

Monosubstituted cyanoacetic esters, obtained by condensation of 1-bromo-3(*s*)-phenoxypropanes (*s* = *o*-Cl, *o*-Br, *o*-I, *o*-, *m*-, and *p*-NO_2) or 1-bromo-2(*s*)-phenoxyethanes (*s* = *o*-Cl, *o*-Br, *o*-I, and *m*-NO_2) with ethyl cyanoacetate by means of potassium carbonate, were transformed through a Curtius degradation into cyanoacetisocyanates. These compounds by hydrolysis in acid or alkaline medium gave α-amino acids. However, hydrolysis of the corresponding carbobenzyloxy- or carbethoxyaminonitriles afforded better yields. The carbobenzyloxyaminonitriles were more readily hydrolyzed in aqueous hydrochloric acid than the carbethoxyaminonitriles. Moreover, the mild action of dry hydrochloric acid on the carbobenzyloxy derivatives yielded the α-amino acids readily whereas similar treatment of the carbethoxy derivatives gave the carbethoxyamino acids.

INTRODUCTION

Synthesis of α-amino acids from ethyl cyanoacetate is possible through the Curtius degradation, $RCO_2Et \rightarrow RCON_3 \rightarrow RNH_2$, as suggested by Darapsky and Hillers in 1915 (3). This method was used many times (4, 5, 6, 7, 8, 9, 10, 11) and was found of wide application even if the yields were occasionally rather low.

The purpose of the present work was to synthesize, from ethyl cyanoacetic ester, new α-amino acids substituted by phenoxyalkyl groups. Modifications of the Darapsky method were also studied in the hope of decreasing the over-all time of reaction and of obtaining better yields in a larger number of cases.

The starting materials, the phenoxyalkylbromides, were prepared with good yields by a process adapted from Marvel and Tanenbaum (12) using sodium phenolates and dibromides in water.

From the halides the monosubstituted cyanoacetic esters were obtained by the use of potassium carbonate (13, 14) instead of sodium ethylate as condensing agent.

The cyanoacethydrazides derived from the cyanoacetic esters were transformed by diazotation into azides. The yields varied from 75 to 85%, as measured by evolution of nitrogen when the azides were decomposed into cyanoacetisocyanates by heat. The temperature of rearrangement was about 50–60°C. These were converted into amino acids or carbamates (urethanes).

Several hydrolytic media were tried with the carbamates. It was found that the original procedure outlined by Darapsky and co-workers could be advantageously modified by using benzyl carbamates. The benzyl carbamates could be hydrolyzed by dry hydrochloric acid in ethyl alcohol, according to the method described by Barkdoll and Ross (2) for carbobenzyloxy groups, and then submitted to a short treatment by aqueous alkali to saponify the orthoester group formed from the nitrile. This process, besides being mild and rapid, is efficient and could be very useful when applied to unstable or slightly soluble carbamates.

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Contribution from the Department of Chemistry, Laval University, Quebec, Que. This paper constitutes part of a thesis submitted to the Graduate School, Laval University, in partial fulfillment of the requirements for the degree of Doctor of Science.
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[3]*Holder of a Price Bursary, and later of a Canadian Industries Limited Research Scholarship.*

TABLE I

BROMIDES, R-Br

Compound	Time, hr.	B.p., °C./mm.	M.p., °C.	Yield, %	n_D^{25}	Formula	Halogens, %	
							Calc.	Found
o-$ClC_6H_4O(CH_2)_3Br$	5–5½	118–120/5	—	68–70	1.555	$C_9H_{10}BrClO$	46.23	45.8
o-$BrC_6H_4O(CH_2)_3Br$	5½	110–115/1–2	—	73–75	1.575	$C_9H_{10}Br_2O$	54.37	53.3
o-$IC_6H_4O(CH_2)_3Br$	5	150–152/3[1]	21–22	80–82	1.611	$C_9H_{10}BrIO$	60.65	60.2
o-$NO_2C_6H_4O(CH_2)_3Br$	10	138–140/1	37–38	58–60	—	$C_9H_{10}BrNO_3$	30.73	30.5
m-$NO_2C_6H_4O(CH_2)_3Br$	7–9	143–148/1[2]	—	72–74	—	$C_9H_{10}BrNO_3$	30.73	30.3
p-$NO_2C_6H_4O(CH_2)_3Br$	6–8	175–177/2	58–59	67–69	—	$C_9H_{10}BrNO_3$	30.73	30.7
o-$ClC_6H_4O(CH_2)_2Br$	16	138–140/12[3]	10–11	64–66	1.565	C_8H_8BrClO	48.99	48.9
o-$BrC_6H_4O(CH_2)_2Br$	20	110–111/1–2[4]	34–35[5]	72–74	—	$C_8H_8Br_2O$	57.10	57.2
o-$IC_6H_4O(CH_2)_2Br$	18	125–130/1–2	48–49[6]	75–77	—	—	—	—
m-$NO_2C_6H_4O(CH_2)_2Br$	30–35	140–145/1–2	39–40[7]	70–72	—	—	—	—

(1) *B.p.: 154–156°C. at 0.2 mm., yield: 59%, Ref. (16).*
(2) *B.p. 186–188°C. at 7 mm., yield: 75%, n_D^{25}: 1.5700, Ref. (18).*
(3) *B.p.: 140–142°C. at 13 mm., yield: 35%, Ref. (15).*
(4) *B.p.: 160–162°C. at 16 mm., Ref. (15).*
(5) *M.p.: 35–36°C., Ref. (1).*
(6) *M.p.: 50–51°C., yield: 34%, Ref. (16).*
(7) *M.p.: 39°C., Ref. (17).*

EXPERIMENTAL

Substituted Phenoxyalkyl Bromides

(*a*) *Phenoxypropyl Bromides*

Mixtures containing 1,3-dibromopropane (1.0 mole), substituted phenol (0.5 mole), sodium hydroxide (0.5 mole), and water (400 ml.) were heated under reflux with mechanical stirring until neutral. More sodium hydroxide (0.15–0.2 mole) was then added and refluxing was continued. After cooling, the neutral mixtures were made alkaline, decanted and extracted if necessary, and, after rapid drying, they were fractionated by distillation under reduced pressure.

(*b*) *Phenoxyethyl Bromides*

The same method as above mentioned was used except that the 1,2-dibromoethane was substituted for 1,3-dibromopropane. From 0.2–0.25 mole of sodium hydroxide was added to each mixture, after neutralization.

The yield of each preparation was based on the initial quantity of the phenol.

The physical properties and yields of the bromides are listed in Table I.

Monosubstituted Cyanoacetic Esters

(*a*) *From 1-Bromo-3-phenoxypropane*

Anhydrous potassium carbonate (0.12 mole) was added to solutions of the suitable bromides (0.10 mole) in ethyl cyanoacetate (0.5 mole). The mixtures were boiled for two and one-half–three hours under a 15–20 mm. pressure on an oil bath usually kept at 115–120°C. Agitation was ensured by introducing a fine stream of dry air through a capillary. After cooling, the mixtures were poured into water (100–150 ml.), extracted with ether, and the combined extracts dried over sodium sulphate. The monosubstituted esters were separated by distillation under reduced pressure. They could be crystallized from ethanol.

(*b*) *From 1-Bromo-2-phenoxyethanes*

In this case, the procedure was as above except that calcium sulphate (10 gm.) was added to the reaction mixtures (to reduce the saponification) and was filtered off before decanting the ethereal extracts.

The yields and properties of the esters are given in Table II.

TABLE II

MONOSUBSTITUTED CYANOACETIC ESTERS, $RCH(CN)COOC_2H_5$

R	B.p., °C./mm.	M.p., °C.	Yield, %	Formula	Calc., %	Found, %
o-$ClC_6H_4O(CH_2)_3$	128–133/0.05	49–50	89–91	$C_{14}H_{16}ClNO_3$	Cl, 12.59	Cl, 12.3
o-$BrC_6H_4O(CH_2)_3$	140–145/0.05	57–58	87–91	$C_{14}H_{16}BrNO_3$	Br, 24.50	Br, 24.2
o-$IC_6H_4O(CH_2)_3$	150–153/0.05	38–39	89–92	$C_{14}H_{16}INO_3$	I, 34.01	I, 34.2
o-$NO_2C_6H_4O(CH_2)_3$	175–180/0.05	76	81–85	$C_{14}H_{16}N_2O_5$	N, 9.54	N, 9.5
m-$NO_2C_6H_4O(CH_2)_3$	160–165/0.05	64	78–81	$C_{14}H_{16}N_2O_5$	N, 9.54	N, 9.4
p-$NO_2C_6H_4O(CH_2)_3$	185–190/0.05	60	75–80	$C_{14}H_{16}N_2O_5$	N, 9.54	N, 9.5
o-$ClC_6H_4O(CH_2)_2$	118–123/0.05	34–35	75–80	$C_{13}H_{14}ClNO_3$	Cl, 13.25	Cl, 13.1
o-$BrC_6H_4O(CH_2)_2$	130–135/0.05	28–29	77–80	$C_{13}H_{14}BrNO_3$	Br, 25.60	Br, 25.7
o-$IC_6H_4O(CH_2)_2$	135–140/0.05	36–37	77–80	$C_{13}H_{14}INO_3$	I, 35.33	I, 35.6
m-$NO_2C_6H_4O(CH_2)_2$	190–195/1–2	64	61–65	$C_{13}H_{14}N_2O_5$	N, 10.03	N, 10.0

Cyanoacethydrazides

The cyanoacetic esters (0.1 mole), fused and slightly cooled, were mixed with hydrazine hydrate (100%, 5 ml.) and ethanol (2–3 ml.). The solutions obtained were placed in an evacuated desiccator over phosphorus pentoxide and the solid products formed were triturated with ethyl ether and water.

The yields of pure hydrazides recrystallized from dilute ethanol varied from 80 to 90%.

Their properties are shown in Table III.

TABLE III
CYANOACETHYDRAZIDES, $RCH(CN)CONHNH_2$

R	M.p., °C.	Formula	Calc., %	Found, %
o-$ClC_6H_4O(CH_2)_3$	87–88	$C_{12}H_{14}ClN_3O_2$	Cl, 13.25	Cl, 13.1
o-$BrC_6H_4O(CH_2)_3$	99–100	$C_{12}H_{14}BrN_3O_2$	Br, 25.60	Br, 25.7
o-$IC_6H_4O(CH_2)_3$	113–114	$C_{12}H_{14}IN_3O_2$	I, 35.33	I, 35.4
o-$NO_2C_6H_4O(CH_2)_3$	112–113	$C_{12}H_{14}N_4O_4$	N, 20.13	N, 20.0
m-$NO_2C_6H_4O(CH_2)_3$	120	$C_{12}H_{14}N_4O_4$	N, 20.13	N, 20.0
p-$NO_2C_6H_4O(CH_2)_3$	127–128	$C_{12}H_{14}N_4O_4$	N, 20.13	N, 20.2
o-$ClC_6H_4O(CH_2)_2$	88–89	$C_{11}H_{12}ClN_3O_2$	Cl, 13.97	Cl, 14.0
o-$BrC_6H_4O(CH_2)_2$	95–96	$C_{11}H_{12}BrN_3O_2$	Br, 26.80	Br, 26.7
o-$IC_6H_4O(CH_2)_2$	97–98	$C_{11}H_{12}IN_3O_2$	I, 36.77	I, 36.9
m-$NO_2C_6H_4O(CH_2)_2$	101–102	$C_{11}H_{12}N_4O_4$	N, 21.21	N, 20.7

Cyanoacetisocyanates and Carbamates

The hydrazides dissolved in dilute hydrochloric acid (2*N*) were diazotized in the usual way. The azides, on formation, were extracted with ethyl ether or chloroform. The combined extracts were washed with water and dried over sodium sulphate and calcium sulphate. The cyanoacetisocyanates were obtained by heating the azides in the presence of toluene or immediately transformed into the carbamates in the presence of benzyl or ethyl alcohol. When purified by washing with sodium hydroxide (5%) and water, the carbamates could be crys-

TABLE IV
CARBAMATES, $RCH(R')NHCOOR''$

R	R′	R″	M.p., °C.	Formula	Calc., %	Found, %
o-$ClC_6H_4O(CH_2)_3$	CN	C_2H_5	46–47	$C_{14}H_{17}ClN_2O_3$	Cl, 11.95	Cl, 11.8
	COOH	C_2H_5	89–90	$C_{14}H_{18}ClNO_5$	Cl, 11.24	Cl, 11.2
o-$BrC_6H_4O(CH_2)_3$	CN	C_2H_5	67–68	$C_{14}H_{17}BrN_2O_3$	Br, 23.45	Br, 23.5
	COOH	C_2H_5	101–102	$C_{14}H_{18}BrNO_5$	Br, 22.20	Br, 21.9
	CN	$CH_2C_6H_5$	84–85	$C_{19}H_{19}BrN_2O_3$	Br, 19.83	Br, 19.8
o-$IC_6H_4O(CH_2)_3$	CN	$CH_2C_6H_5$	90–92	$C_{19}H_{19}IN_2O_3$	I, 28.23	I, 28.3
o-$NO_2C_6H_4O(CH_2)_3$	CN	C_2H_5	66	$C_{14}H_{17}N_3O_5$	N, 13.68	N, 13.6
	COOH	C_2H_5	123–124	$C_{14}H_{18}N_2O_7$	N, 8.60	N, 8.8
m-$NO_2C_6H_4O(CH_2)_3$	CN	C_2H_5	108	$C_{14}H_{17}N_3O_5$	N, 13.68	N, 13.6
p-$NO_2C_6H_4O(CH_2)_3$	CN	C_2H_5	62–63	$C_{14}H_{17}N_3O_5$	N, 13.68	N, 13.5
	CN	$CH_2C_6H_5$	75–76	$C_{19}H_{19}N_3O_5$	N, 11.38	N, 11.4
o-$ClC_6H_4O(CH_2)_2$	CN	C_2H_5	69	$C_{13}H_{15}ClN_2O_3$	Cl, 12.56	Cl, 12.7
o-$BrC_6H_4O(CH_2)_2$	CN	C_2H_5	75	$C_{13}H_{15}BrN_2O_3$	Br, 24.45	Br, 24.2
o-$IC_6H_4O(CH_2)_2$	CN	C_2H_5	74	$C_{13}H_{15}IN_2O_3$	I, 33.95	I, 33.7
	CN	$CH_2C_6H_5$	91–92	$C_{18}H_{17}IN_2O_3$	I, 29.09	I, 28.8
m-$NO_2C_6H_4O(CH_2)_2$	CN	$CH_2C_6H_5$	83–84	$C_{18}H_{17}N_3O_5$	N, 11.83	N, 11.9

TABLE V

AMINO ACIDS AND DERIVATIVES

Amino acids R-CH(NH)$_2$COOH R	Starting materials R-CH(CN)R′ R′	Hydrolyzing method: Agent	Temp., °C.	Time, hr.	Yield, %	Formula	Calc., %	Found, %	Derivatives	M.p., °C.	Formula	Calc., %	Found, %
o-ClC$_6$H$_4$O(CH$_2$)$_3$	NHCOOC$_2$H$_5$	37% HCl	100	2	63–67	$C_{11}H_{14}ClNO_3$	Cl, 14.57	Cl, 14.4	Phenylureido	169–171*	$C_{18}H_{19}ClN_2O_4$	Cl, 9.78	Cl, 9.6
		37% HCl	120	1½	65–69								
o-BrC$_6$H$_4$O(CH$_2$)$_3$	NCO	30% KOH[1]	B.p.	½	43–47	$C_{11}H_{14}BrNO_3$	Br, 27.73	Br, 27.8	Phenylureido	178–180*	$C_{18}H_{19}BrN_2O_4$	Br, 19.65	Br, 19.5
	NHCOOC$_2$H$_5$	16% HCl	B.p.	72	65–69								
		37% HCl	120	1½	65–69								
	NHCOOCH$_2$C$_6$H$_5$	16% HCl	B.p.	9	72–78								
		Dry HCl[2]	B.p.	1–1½	68–72								
o-IC$_6$H$_4$O(CH$_2$)$_3$	NHCOOCH$_2$C$_6$H$_5$	Dry HCl	B.p.	1–1½	67–71	$C_{11}H_{14}INO_3$	I, 37.86	I, 37.4	Phenylureido	184–186*	$C_{18}H_{19}IN_2O_4$	I, 27.95	I, 28.0
o-NO$_2$C$_6$H$_4$O(CH$_2$)$_3$	NHCOOC$_2$H$_5$	16% HCl	B.p.	60	76–81	$C_{11}H_{14}N_2O_5$	N, 11.02	N, 11.0	Phenylureido	163–165*	$C_{18}H_{19}N_3O_6$	N, 11.26	N, 11.3
		37% HCl	100	4	76–81								
		37% HCl	120	1½	72–78								
m-NO$_2$C$_6$H$_4$O(CH$_2$)$_3$	NCO	37% HCl[3]	B.p.	1	29–31	$C_{11}H_{14}N_2O_5$	N, 11.02	N, 11.0	Hydantoin	157–158	$C_{12}H_{13}N_3O_5$	N, 15.05	N, 15.2
									Phenylureido	174–176*	$C_{18}H_{19}N_3O_6$	N, 11.26	N, 11.3
	NHCOOC$_2$H$_5$	16% HCl	B.p.	60	72–77								
		37% HCl	100	3	77–83								
		37% HCl	120	1½	75–81								
p-NO$_2$C$_6$H$_4$O(CH$_2$)$_3$	NHCOOC$_2$H$_5$	37% HCl	120	1½	69–73	$C_{11}H_{14}N_2O_5$	N, 11.02	N, 10.7	Phenylureido	190–192*	$C_{18}H_{19}N_3O_6$	N, 11.26	N, 11.4
	NHCOOCH$_2$C$_6$H$_5$	37% HCl	100	1½	66–70								
o-ClC$_6$H$_4$O(CH$_2$)$_2$	NHCOOC$_2$H$_5$	37% HCl	120	1½	60–64	$C_{10}H_{12}ClNO_3$	Cl, 15.45	Cl, 15.5	Phenylureido	167–169*	$C_{17}H_{17}ClN_2O_4$	Cl, 10.18	Cl, 10.1
		Ba(OH)$_2$[4]	160	½	40–46								
o-BrC$_6$H$_4$O(CH$_2$)$_2$	NHCOOC$_2$H$_5$	16% HCl	B.p.	72	48–52	$C_{10}H_{12}BrNO_3$	Br, 29.17	Br, 28.9	*p*-Tolylsulphonamido	147–148	$C_{16}H_{18}BrNO_5S$	Br, 18.66	Br, 18.8
		37% HCl	100	2	57–61								
o-IC$_6$H$_4$O(CH$_2$)$_2$	NHCOOCH$_2$C$_6$H$_5$	Dry HCl	B.p.	1–1½	59–63	$C_{10}H_{12}INO_3$	I, 39.53	I, 40.0	*p*-Tolylsulphonamido	157–158	$C_{16}H_{18}INO_5S$	I, 26.72	I, 27.0
m-NO$_2$C$_6$H$_4$O(CH$_2$)$_2$	NHCOOCH$_2$C$_6$H$_5$	Dry HCl	B.p.	1–1½	58–62	$C_{10}H_{12}N_2O_5$	N, 11.67	N, 11.3	Copper salt	—	$C_{20}H_{22}CuN_4O_{10}$	Cu, 11.75	Cu, 11.5

[1] *Aqueous solution. Mixture left standing for 12 hr. at 25°C. after boiling.*
[2] *Treatment followed by a mild hydrolysis with aqueous potassium hydroxide.*
[3] *Heated slowly in an open vessel. Mixture left standing for 8 hr. at 25°C. after boiling.*
[4] *Hot saturated aqueous solution.*
* *Dec. m.p. determined with Dennis & Shelton apparatus.*

tallized from a mixture of petroleum ether (65–110°C.) and ethyl ether. By hydrolysis of the nitrile group in hot alcoholic solution with dry hydrochloric acid, followed by a mild alkaline treatment, the carbethoxyaminonitriles gave rise to carbethoxyamino acids.

The properties of the carbamates are listed in Table IV.

Amino Acids

The crude carbamates or cyanoacetisocyanates were hydrolyzed. The acidified mixtures were evaporated to dryness. Baryta, when used, was first eliminated by sulphuric acid. Water or ethanol was added to the residues. After filtration, the solutions were purified in the usual way. Finally, the amino acids were precipitated with ammonia or sodium hydroxide when in aqueous media, or with piperidine when in alcoholic media. The yields were based on the quantities of cyanoacetisocyanates or carbamates contained in the crude materials.

A summary of the methods of hydrolysis used together with the properties of the derivatives of the amino acids is given in Table V.

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LYCOPODIUM ALKALOIDS

I. PHYSICAL PROPERTIES AND X-RAY CRYSTALLOGRAPHIC DATA OF SOME *LYCOPODIUM* ALKALOIDS[1]

By Osvald Knop and David B. MacLean

ABSTRACT

Physical properties and X-ray crystallographic data of three major alkaloids of the *Lycopodium* group and of some of their hydrohalide salts were determined. The hydrobromide and hydroiodide of annotinine were shown to be isomorphous and therefore suitable for a detailed investigation by X-ray diffraction methods.

Some 39 alkaloids have already been isolated from species of the *Lycopodium* family. Of these, only two, annotinine and lycopodine, have been subjected to a chemical structural investigation. In both cases, the structural problem has not advanced further than an examination and establishment of the nature of the functional groups present. Despite considerable study little is known of the ring structure of either of these alkaloids.

The present investigation was initiated to determine the possibility of furthering the structure determination by X-ray diffraction methods. For this purpose, the three major alkaloids of *Lycopodium annotinum* L. reported by Manske and Marion (3), annotinine, obscurine, and lycopodine, were isolated, purified, and crystallized in a form suitable for X-ray examination. Hydrohalide salts of annotinine and lycopodine were prepared, since the introduction of a heavy atom is a possible route to a complete crystal structure determination. Moreover, it was hoped that some of these salts would be isomorphous and thus further facilitate an X-ray study by Fourier methods. Crystalline hydrohalide salts of obscurine could not be obtained despite repeated attempts to effect crystallization from various organic solvents.

The structures of a family of alkaloids usually exhibit a generic relationship, and the solution of the structure of one of the alkaloids aids in the investigation of the other members. It was, therefore, hoped that a compound suitable for a complete structure determination would be found among the compounds mentioned above. The results of a survey made of the properties of these substances are reported in Tables I and II

Densities were measured in order to determine the number of asymmetric formula units per unit cell, Z. The experimental values of the densities reported in Table I are in fair agreement with the values calculated from the unit cell dimensions and the values of Z.

In Table I, it may be noted that annotinine hydrochloride crystallizes with one-half molecule of water. It cannot, then, be isomorphous with the hydrobromide or hydroiodide. Also, considerable difficulty was encountered in the

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TABLE I

FORMULAE AND PROPERTIES OF THE ALKALOIDS AND THEIR SALTS

Compound	Formula	M.p.(corr.), °C.	Density	
			Experimental	Calculated
Annotinine	$C_{16}H_{21}O_3N$	232	1.28	1.324 ± 0.014
Annotinine hydrochloride	$C_{16}H_{21}O_3N.HCl.\frac{1}{2}H_2O$	210–211 (decomp.)	1.38	—
Annotinine hydrobromide	$C_{16}H_{21}O_3N.HBr$	260–263 (decomp.)	1.53	1.556 ± 0.014
Annotinine hydroiodide	$C_{16}H_{21}O_3N.HI$	237–239 (decomp.)	1.66	1.664 ± 0.015
Lycopodine	$C_{16}H_{23}ON$	116	1.18	1.138 ± 0.010
Lycopodine hydrobromide	$C_{16}H_{23}ON.HBr$	360	1.40	1.382 ± 0.011
Obscurine	$C_{18}H_{28}ON_2$	285–287 (slight darkening)	1.24	1.297 ± 0.012

The formulae were established by microanalysis of assorted crystals from the same crystallization batches from which single crystals for X-ray work were selected.

preparation of satisfactory single crystals of this salt. For these reasons, annotinine hydrochloride hemihydrate was excluded from the X-ray work.

The crystal symmetry and unit cell dimensions of the compounds are recorded in Table II. These properties were determined by X-ray diffraction methods using well-developed single crystals. The largest dimension of the crystals selected did not exceed 0.3 mm. In order to minimize the effect of absorption of X-rays in the crystal, the crystals were ground to a cylindrical shape.

TABLE II

CRYSTAL SYMMETRY AND UNIT CELL DIMENSIONS OF THE ALKALOIDS AND THEIR SALTS

Compound	Space group	Unit cell dimensions, Å			Volume of the u.c., Å³	Z
		a	b	c		
Annotinine	$P2_12_12_1$	9.09 ± 0.04	10.52 ± 0.03	14.42 ± 0.04	1379 ± 14	4
Annotinine hydrobromide	$P2_12_12_1$	8.15 ± 0.02	10.94 ± 0.04	17.04 ± 0.04	1519 ± 13	4
Annotinine hydroiodide	$P2_12_12_1$	8.06 ± 0.03	11.16 ± 0.03	17.88 ± 0.04	1608 ± 14	4
Lycopodine	$P2_12_12_1$	8.81 ± 0.03	11.07 ± 0.03	28.36 ± 0.08	2766 ± 25	8
Lycopodine hydrobromide	$P2_12_12_1$	7.70 ± 0.02	12.72 ± 0.02	15.61 ± 0.04	1529 ± 12	4
Obscurine	$P2_12_12_1$	7.35 ± 0.03	12.81 ± 0.02	15.66 ± 0.04	1474 ± 13	4

The diameter of the cylinders was, on the average, not greater than 0.1 mm. Rotation and equi-inclination Weissenberg photographs were taken with filtered Cu*K*-radiation ($\lambda_{aver.} = 1.54180$ Å, $\lambda_{a_1} = 1.54052$ Å) from a hot-cathode X-ray tube in a camera of 57.3 mm. diameter. Filtered Fe*K*-radiation ($\lambda_{aver.} = 1.93731$ Å, $\lambda_{a_1} = 1.93600$ Å) was used occasionally to record low-angle reflections not obtainable with copper radiation.

The unit cell dimensions were determined from rotation and zero-level Weissenberg photographs taken about at least two crystallographic axes. The uncorrected values were plotted against the function $\frac{1}{2}\left(\frac{\cos^2\theta}{\sin\theta} + \frac{\cos^2\theta}{\theta}\right)$, given by Nelson and Riley (4), and the straight lines so obtained extrapolated to $\theta = 0$ to give the final values reported in Table II.

The crystal symmetry was obtained from Laue photographs and from morphological evidence. The space groups were determined from systematic absences of reflections on a sufficient number of Weissenberg photographs, and by a consideration of the number of asymmetric formula units per unit cell.

The results of the preliminary X-ray investigations are listed in Table II. All the compounds examined are orthorhombic and crystallize with the same space group, D_2^4–$P2_12_12_1$. This space group is determined uniquely by the systematic extinctions of the ($h00$), ($0k0$), and ($00l$) reflections with h, k, and l odd. The general and the only position in this space group is fourfold, and four asymmetric units are, therefore, required per unit cell. Since lycopodine contains eight formula units per unit cell, it has to be assumed that two formula units of lycopodine are associated in the crystal to an asymmetric unit. That the formula unit of lycopodine is $C_{16}H_{25}ON$ and not $C_{32}H_{50}O_2N_2$ was confirmed by the cryoscopic determination of the molecular weight of lycopodine in benzene and by the existence of a hydrobromide, $C_{16}H_{25}ON.HBr$. The formula unit of the hydrobromide follows directly from the X-ray data in Table II. It is suggested that the dimerization of lycopodine in the crystalline state might be due to the formation of a crystalline *dl*-compound. As had been expected, there is no evidence from these data suggesting any particular shape of the alkaloid molecules.

Upon consideration of the X-ray data and of the available chemical information annotinine hydrobromide was selected for a detailed study. More information is available on the structure of annotinine than on the other alkaloids and the problem has been under active chemical investigation in these laboratories (5). Moreover, the identical space group and the close similarity of the unit cell dimensions of annotinine hydrobromide and hydroiodide suggest that these two compounds might be isomorphous. This was confirmed by Patterson $P(vw)$ projections for the hydrobromide and hydroiodide, from which it was found that the positions of the corresponding self-consistent peaks, in terms of fractional coordinates, were identical for both compounds. Details of this work along with further investigations by Fourier methods will be communicated in a subsequent paper dealing with an attempt to determine the crystal structure of annotinine hydrobromide. From consideration of the X-ray data alone lycopodine hydrobromide is equally as suitable for further work as annotinine hydrobromide.

Lycopodine was, however, of less immediate interest to the authors and an intensive study of its salts was, therefore, not undertaken at this time. Because of the unavailability of crystalline hydrohalide salts, obscurine could not be considered for further work.

EXPERIMENTAL

The source of the alkaloids studied in this paper was *Lycopodium annotinum* L. The plant was collected in Halifax County, Province of Nova Scotia, during the summer months of 1950 and 1951. The alkaloids were extracted from the dried material and isolated in the manner described by Manske and Marion (2, 3). The free bases were purified by repeated crystallization from organic solvents. Well-developed small single crystals were then selected from the individual crystallization batches for microanalysis, melting-point and density determinations, and X-ray work. All the substances described below crystallized in well-developed colorless crystals of high brilliancy.

All melting points were corrected. Densities were determined by the flotation method, in which mixtures of various organic liquids or concentrated aqueous solutions of inorganic salts were used as flotation liquids. The calculated densities were obtained from the unit cell dimensions of the individual compounds in the usual manner.

Annotinine

Annotinine was recrystallized twice from a mixture of methanol and chloroform (1 : 1). It melted at 232°C. in agreement with the recorded value (3). Calc. for $C_{16}H_{21}O_3N$: C, 69.82; H, 7.63; N, 5.09%. Found: C, 69.79, 69.99; H, 7.34, 7.42; N, 5.33, 5.36%. The density was determined with a concentrated aqueous solution of calcium chloride as flotation liquid because of the appreciable solubility of annotinine in organic solvents of suitable density. Although small well-developed crystals without any apparent imperfection were used in this determination, the value obtained, 1.28, was not in very good agreement with the calculated density, 1.324. This discrepancy is probably due to microscopic air bubbles in the concentrated flotation solution. The pycnometric method with decalin as the displacement liquid gave a slightly higher value, 1.30.

Lycopodine

This base was recrystallized three times from petroleum ether. It melted at 116°C. in agreement with the recorded value (2). Calc. for $C_{16}H_{25}ON$: C, 77.73; H, 10.12%. Found: C. 78.24, 78.15; H, 10.29, 10.20%. A concentrated aqueous solution of sodium bromide was employed in the density determination.

Obscurine

Obscurine was recrystallized twice from *n*-propanol. It melted at 285–287°C. (slight darkening). The recorded value is 282°C. (3). Calc. for $C_{18}H_{28}ON_2$: C, 75.00; H, 9.72; N, 9.72%. Found: C, 74.39, 74.50; H, 9.58, 9.64; N, 10.17, 10.23%. The density was determined with a mixture of benzene and carbon tetrachloride as flotation liquid.

Annotinine Hydrochloride Hemihydrate

A solution of annotinine in acetone was made just acid to Congo red with concentrated aqueous hydrochloric acid. The separated hydrochloride was recrystallized four times from a mixture of methanol and acetone (1 : 3). It melted at 210–211°C. (decomposition). Calc. for $C_{16}H_{21}O_3N.HCl.\frac{1}{2}H_2O$: C, 59.80; H, 7.16; N, 4.36%. Found: C, 60.00, 59.87; H, 7.57, 7.35; N, 4.34, 4.44%. The density was determined with a mixture of benzene and carbon tetrachloride as flotation liquid. Considerable difficulty was experienced in attempts to obtain suitable single crystals for X-ray work as the salt crystallized in agglomerates of very small crystals.

Annotinine Hydrobromide

This salt was prepared in a manner similar to the hydrochloride. After two recrystallizations from methanol it melted at 260–263°C. (decomposition). Calc. for $C_{16}H_{21}O_3N.HBr$: C, 53.95; H, 6.18; N, 3.93%. Found: C, 54.31, 54.14; H, 6.17, 6.07; N, 4.48, 4.66%. The density was determined with a mixture of ethylene trichloride and carbon tetrachloride as flotation liquid.

Annotinine Hydroiodide

The hydroiodide was prepared in a manner similar to the hydrochloride. After four recrystallizations from methanol it melted at 237–239°C. (decomposition). Calc. for $C_{16}H_{21}O_3N.HI$: C, 47.65; H, 5.46; N, 3.47%. Found: C, 47.71, 48.02; H, 5.55, 5.47; N, 3.50, 3.49%. The density was determined with a mixture of carbon tetrachloride and iodobenzene as flotation liquid.

Lycopodine Hydrobromide

This salt was prepared by treating a concentrated methanol solution of the base with concentrated aqueous hydrobromic acid. After two recrystallizations from methanol it melted at 360°C. in agreement with the recorded value (1). Calc. for $C_{16}H_{25}ON.HBr$: C, 58.53; H, 7.92; N, 4.23%. Found: C, 58.81, 58.93; H, 8.08, 8.12; N, 4.40, 4.24%. The density was determined with a mixture of benzene and carbon tetrachloride as flotation liquid.

ACKNOWLEDGMENTS

The authors wish to express their thanks to Prof. G. V. Douglas, Head of the Geology Department, Dalhousie University, for the permission to use his North-American Philips X-ray diffraction unit, and to the Nova Scotia Research Foundation for making available a Unicam two-circle optical goniometer and a Unicam Weissenberg goniometer.

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ELECTROPHORETIC HOMOGENEITY OF POLYSACCHARIDES IN MOLAR ALKALI[1]

By J. Ross Colvin, W. H. Cook, and G. A. Adams

ABSTRACT

The usefulness of electrophoretic patterns of polysaccharides in molar alkali as a general criterion of the purity of these substances has been studied. No clear separation of the components of a hemicellulose, of wheat starch, or of potato starch could be demonstrated. Similarly, mixtures of wheat starch and inulin, pectin and sodium alginate, laminarin and sodium alginate, inulin and wheat starch plus sodium alginate could not be resolved in 1*M* potassium hydroxide. Nonetheless, mixtures of laminarin and sodium alginate separated rapidly at pH 6.8 and 10.4. Contrary to previous reports, it is shown that the electrophoretic technique cannot be used with confidence as a general criterion of the purity of polysaccharides in molar alkali. However, it may be applicable for the characterization and fractionation of a limited class of polysaccharides which are soluble close to neutrality.

INTRODUCTION

Ionizing polysaccharides should exhibit electrophoretic mobility at pH values in the region of neutrality and this expectation has been confirmed for carrageenin (1), algin (6), and pectin (7). Electrophoretic heterogeneity is common in such materials but clear-cut evidence for the existence of discrete components is comparatively rare. Recently Isherwood and Jermyn (2) reported that electrophoretic examination of a mixture of pear cell wall polysaccharides in 1*M* sodium hydroxide revealed at least as many components as there were monosaccharides shown by paper chromatography of a hydrolyzate. This observation suggests that the differential ionization of the hydroxyl groups of simple polysaccharides in strong alkaline solution may result in sufficiently different mobilities to permit their separation. If so, electrophoretic analysis might provide a much needed criterion of the purity of a polysaccharide and, with adequate component separation, a possible method of fractionation. A number of mixtures of polysaccharides of widely different composition have therefore been examined to see whether such separation is usually observed.

MATERIALS AND METHODS

Preliminary experiments were undertaken with the apparatus, electrodes, cell, and technique described by Longsworth (3) using saturated potassium chloride to protect the electrodes from the strong alkali. Observations were made at 0 ± 0.1°C. and all samples were dialyzed in Visking 18/32 cellulose tubing for 24 hr. against the solvent. Some early measurements on a hemicellulose extracted from wheat straw occasionally yielded patterns that suggested the separation of components but the results were not reproducible and the experimental conditions were considered unsatisfactory. Subsequently, in a private communication, Dr. Isherwood kindly provided details of his experimental conditions. His suggestions included the use of low current densities and other experimental precautions to avoid convection, and the use of large electrodes

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to support the currents for lengthy periods. All subsequent measurements were made with heavy electrodes capable of passing a current of 20 ma. for five days without gassing. Since the density of 1*M* sodium hydroxide has a larger temperature coefficient than 1*M* potassium hydroxide, the latter was used in subsequent experiments in preference to the sodium hydroxide used by Isherwood and Jermyn.

Even after these changes had been made, however, no separable components were observed with a wheat straw hemicellulose containing 9.3% uronic acid anhydride, 72% D-xylose, 14.2% L-arabinose, and 2.6% ash. A typical diagram (Fig. 1*a*) shows evidence of heterogeneity by spreading of the boundary but no clear indication of separable components.

Other polysaccharides used included wheat starch, potato starch, inulin, pectin, sodium alginate, and laminarin. The wheat starch was deproteinized with dilute ammonia and defatted with 80% dioxane. The final fat, protein, and ash contents were 0.18, 0.14, and 0.18 respectively. The laminarin studied was the water-soluble extract from *Laminaria digitata* (4). It was obtained from the Scottish Institute of Seaweeds Research through the kindness of Dr. W. A. P. Black. All the remaining materials were commercial samples and were dissolved in 1*M* potassium hydroxide without further treatment. The starches and inulin were dissolved by heating to 70°C. and yielded a slightly opalescent solution. All solutions were dialyzed except those containing laminarin, which is known to be of low molecular weight (4).

RESULTS

Repeated attempts to separate wheat starch in 1*M* potassium hydroxide into its two components, amylose and amylopectin, failed. Similar attempts to separate a mixture of wheat starch and inulin were also unsuccessful. A typical pattern is shown in Fig. 1*b*. In a subsequent communication Dr. Isherwood advised extending the periods greatly. Accordingly, the results of two consecutive experiments on the starch–inulin mixture, using very long intervals, are shown in Figs. 1*c* and 1*d*. In Fig. 1*c* the ascending boundary indicates separation of two or more components while the descending boundary remains single. However, in Fig. 1*d* the ascending boundary remained extremely sharp while the descending boundary was the only one to indicate splitting. Since, in these experiments a single boundary is more conclusive than one showing two or more peaks, especially after a movement over more than 3.5 cm., these two experiments taken together show that inulin cannot be readily separated from wheat starch electrophoretically. All other experiments on this system supported this conclusion. Fractionation of potato starch in alkaline solution also failed. The results of one experiment using high currents for long periods are given in Fig. 1*e*.

Attempts were then made to resolve mixtures of two different polysaccharides, pectin and sodium alginate. As shown in Fig. 1*f*, there was no evidence of clear-cut separation of the components. Sodium alginate was then added to an inulin – wheat starch mixture with the hope that it might be resolved into at least two

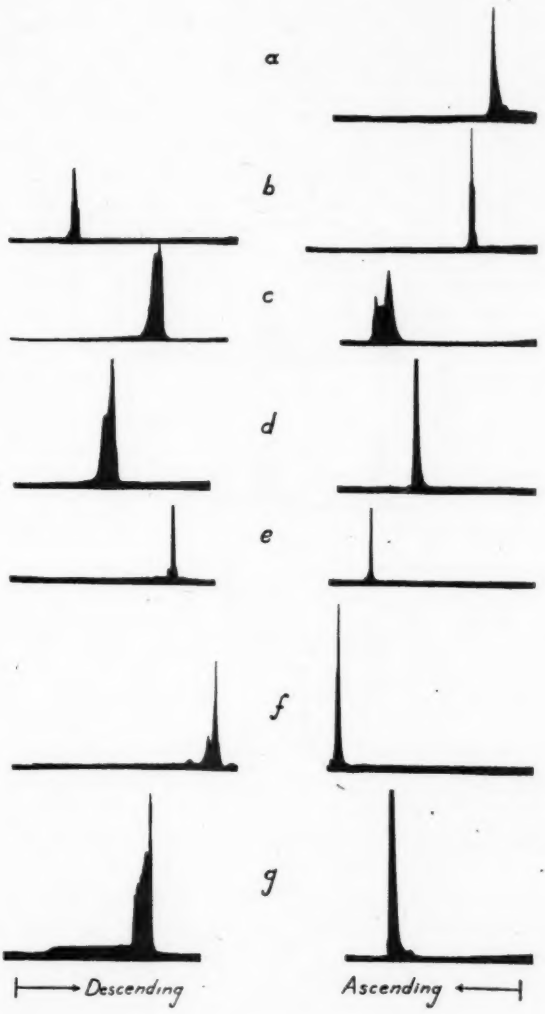

FIG. 1. Electrophoretic patterns of polysaccharides or mixtures of polysaccharides in 1*M* potassium hydroxide.

a. Ascending boundary of 0.5% wheat starch and 0.5% inulin. Time 502 min. Field strength 0.42 volts per cm.
b. 0.5% wheat starch and 0.5% inulin. Time 657 min. Field strength 0.40 volts per cm.
c. 0.5% wheat starch and 0.5% inulin. Time 4600 min. Field strength 0.24 volts per cm.
d. 0.5% wheat starch and 0.5% inulin. Time 5710 min. Field strength 0.26 volts per cm.
e. 0.5% potato starch. Time 2133 min. Field strength 0.57 volts per cm.
f. 0.5% pectin and 0.5% sodium alginate. Time 4480 min. Field strength 0.34 volts per cm.
g. 0.5% inulin and 0.5% wheat starch and 0.5% sodium alginate. Time 3964 min. Field strength 0.26 volts per cm.

fractions. The results, shown in Fig. 1*g*, indicate that it is doubtful that even these widely different polysaccharides can be resolved by this method.

Finally, because it was rather surprising that a highly charged polyelectrolyte such as sodium alginate did not separate from starch and inulin under these conditions, the resolution of laminarin from sodium alginate was attempted over a series of pH's. Laminarin was selected because it is a relatively short chain glucosan analogous to starch (4) and is soluble in neutral buffers. The results are shown in Fig. 2. Obviously the alginate polyion separates extremely rapidly

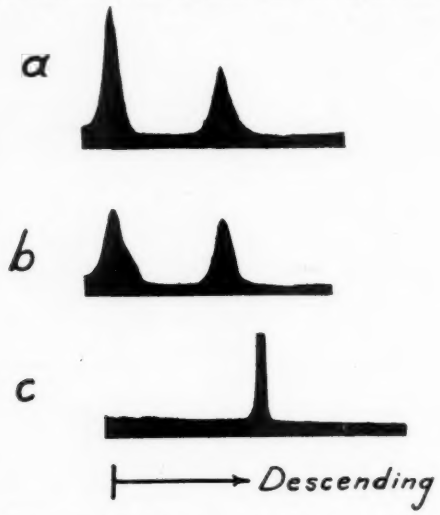

FIG. 2. Electrophoretic patterns of mixtures of laminarin, 0.5%, and sodium alginate, 0.5%, at three pH's.
a. pH 6.8. 0.05*M* Phosphate buffer. Time 27 min. Field strength 8.0 volts per cm.
b. pH 10.4. Buffer, 0.05*M* sodium hydroxide, 0.05*M* glycine, and 0.05*M* sodium chloride. Time 42 min. Field strength 5.0 volts per cm.
c. 1*M* potassium hydroxide. Time 1290 min. Field strength 0.36 volts per cm.

from laminarin at lower pH levels but cannot be resolved in molar alkali. This is not due to any permanent alteration of the alginate for, after neutralization of the base and dialysis, it may be separated from laminarin as readily as before. Furthermore, it seems improbable that the potassium ion has any specific effect on the electrophoretic properties of alginate or other polysaccharides. Such specific effects of this ion on the viscosity of other polysaccharides are known (5) but laminarin and alginate in 1*M* sodium hydroxide gave results similar in all respects to those for the potassium base.

DISCUSSION

The results of these experiments show that the electrophoretic technique applied to polysaccharide mixtures dissolved in strong alkali solutions is not generally useful for their separation nor can the patterns be used with confidence as a general criterion of purity. The separation of polysaccharides of different

structure might reasonably be minimal in molar alkali since the adsorption of the hydroxyl ions on the large neutral molecules would mask any differences in the ionization constants of the sugar hydroxyls. Thus, sodium alginate is readily separated from unionized polysaccharides in the region of neutrality but not in strong alkali.

The high conductivity of these alkali solutions demands long experimental periods. In practice such techniques are time-consuming and subject to error. Low field strengths also reduce the resolving power and during extended periods diffusion may mask any separation that occurs.

The electrophoretic technique may be useful under more restricted conditions. If the polysaccharide mixtures were sufficiently soluble, useful electrophoretic separations might be effected in the region of neutrality. Thus the technique might be used as a criterion of purity in mixtures varying in uronic acid content and under favorable conditions it might even be possible to effect some separation. This possibility has not been investigated yet.

ACKNOWLEDGMENTS

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GARRYA ALKALOIDS

I. THE STRUCTURE OF GARRYINE AND VEATCHINE[1]

By K. Wiesner, S. K. Figdor, M. F. Bartlett,[2] and D. R. Henderson

ABSTRACT

By means of hydrogenation, oxidation, and isomerization experiments it has been established that the chemistry of veatchine and garryine parallels the chemistry of atisine and isoatisine. Dehydrogenation of the *Garrya* alkaloids yields a base $C_{16}H_{15}N$, which, on the basis of comparison of ultraviolet spectra, might be a substituted phenanthridine. 1-Methyl-7-ethyl phenanthrene has also been obtained. The chemistry and structure of the aconite and *Garrya* alkaloids is discussed in the light of the present work.

Oneto (11) has described the isolation of the two alkaloids, veatchine and garryine, from the bark of *Garrya veatchii* Kellogg. They have both been given the empirical formula $C_{22}H_{32}O_2N$. Garryine crystallized as a monohydrate, m.p. ca. 96°C. Veatchine crystallized without water of crystallization and melted at 122-123°C. The separation of these two alkaloids was achieved by fractional precipitation with alkali; the material precipitating first was richer in garryine.

We have achieved a quantitative separation of these alkaloids by a nine-funnel countercurrent distribution between chloroform and an aqueous buffer of pH = 7. In this system veatchine remains in the first three funnels and garryine travels to the last two; in between lies a deep "valley". The properties of both compounds are in fair agreement with those described by Oneto (11) with the exception that both compounds and their derivatives check perfectly to the formula $C_{22}H_{33}O_2N$. Both compounds show approximately one active hydrogen in the Zerevitinoff determination and both have one N-alkyl group.

Potentiometric microtitration in 80% methyl cellosolve shows veatchine to have a pK of 11.5 and garryine a pK of 8.7.

The infrared spectra of the two alkaloids are almost indistinguishable. There is an — OH band at 3300 cm.$^{-1}$ At 1665 cm.$^{-1}$ there is a band which is too weak for a carbonyl group and is likely to be due to a carbon–carbon double bond. A band at 1440 cm.$^{-1}$, which disappears on hydrogenation, is due to a terminal methylene group; the overtone for this group appears at 1800 cm.$^{-1}$ The band at 1400 cm.$^{-1}$ is probably due to — CH_2 bending. One other band, which can be definitely assigned, is that at 1375 cm.$^{-1}$, which is characteristic of a C-methyl group.

Infrared spectra are given in Figs. 1-8.

On hydrogenation with Adams catalyst in glacial acetic acid, both alkaloids absorb two moles of hydrogen and give the same tetrahydro compound $C_{22}H_{37}O_2N$ (m.p. 147-149°C.), characterized also by the picrate. The pK value of tetrahydroveatchine is 6.85. Kuhn-Roth oxidation of tetrahydroveatchine indicates

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Contribution from the Chemistry Laboratories of the University of New Brunswick, Fredericton, N.B.
[2] *Holder of a Beaverbrook Graduate Scholarship.*

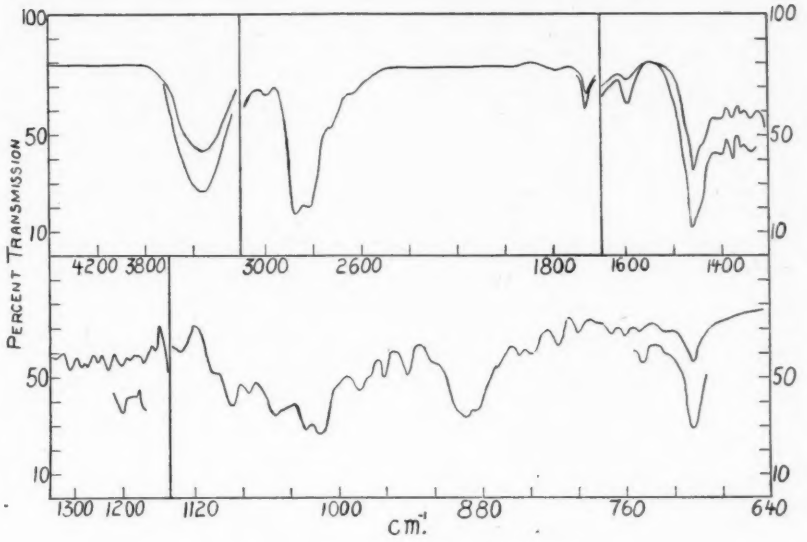


FIG. 1. Infrared spectrum of veatchine.

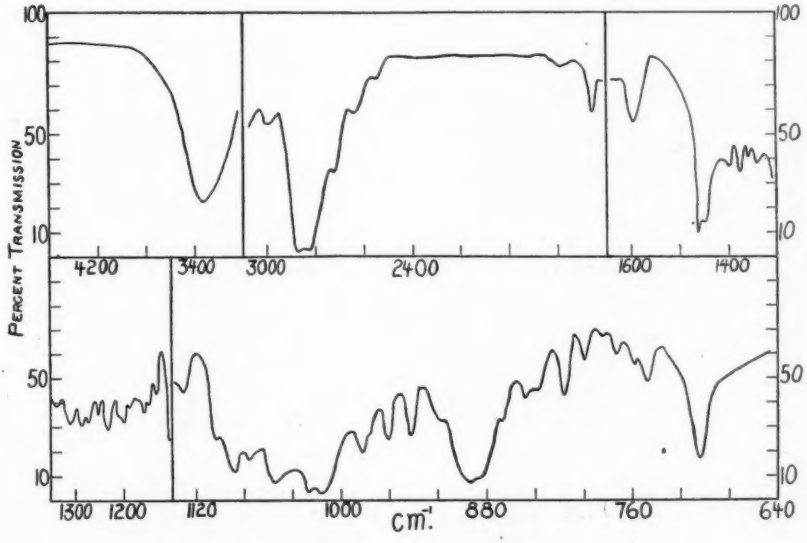


FIG. 2. Infrared spectrum of garryine.

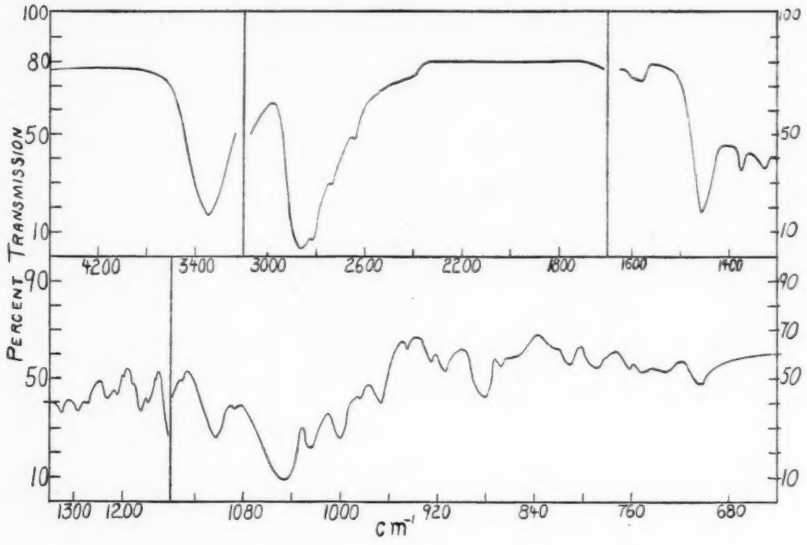


FIG. 3. Infrared spectrum of tetrahydrogarryine.

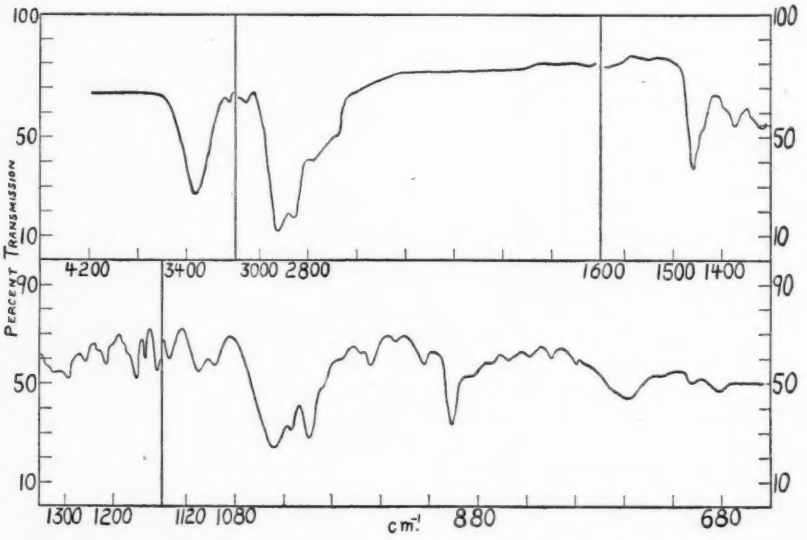


FIG. 4. Infrared spectrum of $LiAlH_4$ reduced veatchine.

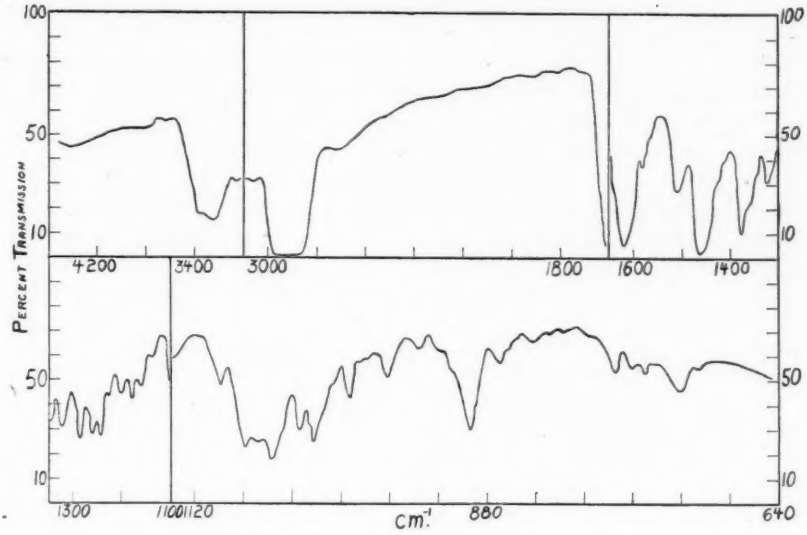


FIG. 5. Infrared spectrum of oxogarryine.

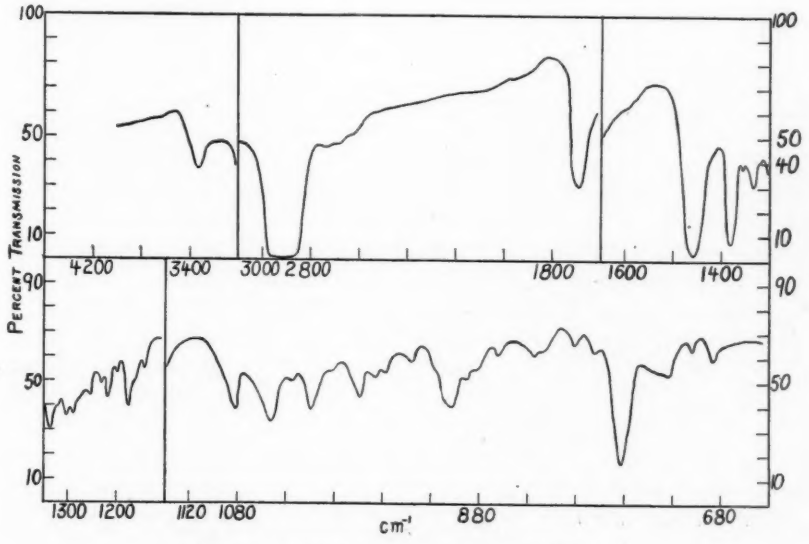


FIG. 6. Infrared spectrum of oxoveatchine *A*.

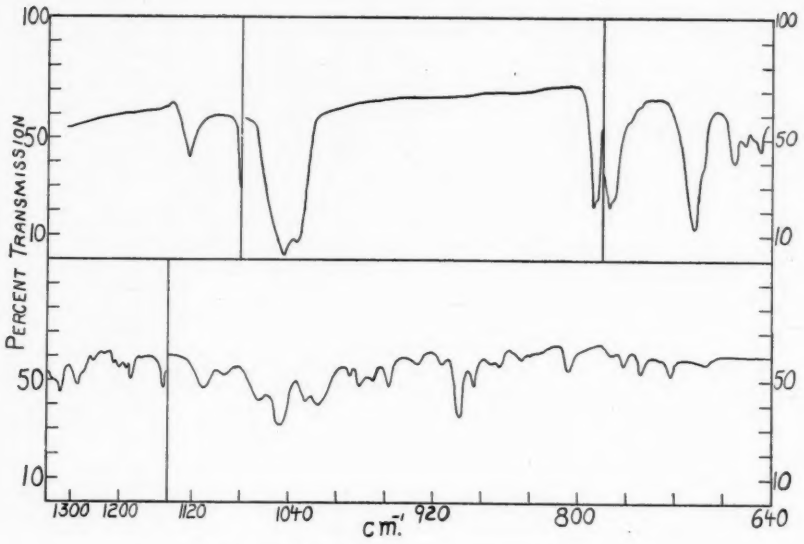


FIG. 7. Infrared spectrum of oxoveatchine *B*.

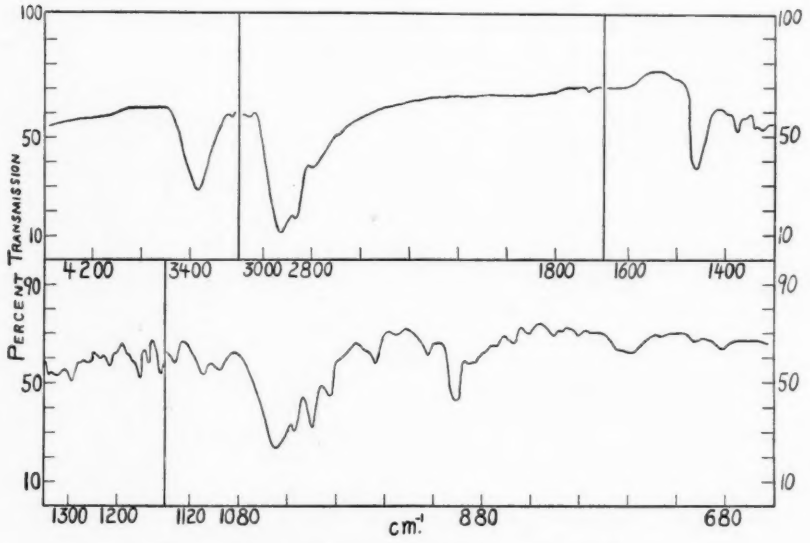


FIG. 8. Infrared spectrum of $LiAlH_4$ reduced oxogarryine.

a content of two C-methyl groups, whereas both veatchine and garryine analyze for only one such group; this result is in agreement with the interpretation of the infrared spectra. The presence of a terminal methylene group in veatchine and garryine was further corroborated by the isolation of formaldehyde as the dimedone complex in ozonolysis experiments.

Veatchine can be quantitatively isomerized to garryine by the action of boiling alcoholic sodium hydroxide.

These results indicate that the difference between garryine and veatchine is just in the position of one carbon–carbon double bond, and this bond must be associated with the nitrogen. It is well established (1) that tertiary bases of the type $> N - \underset{|}{C} = C <$ are unusually strong, because they form salts having the characteristics of quaternary ammonium salts.

Neither garryine nor veatchine contains a carbonyl group, as is seen from the infrared spectra and from the failure to obtain carbonyl derivatives.

Acetylation of veatchine yielded a basic acetyl hydrochloride, which consistently gave analytical values between a mono- and a di-acetyl compound. The free acetylated base analyzed well for a monoacetyl compound.

Tetrahydroveatchine yielded a basic diacetyl derivative on acetylation.

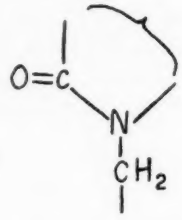
From these results it would seem that the hydrogenation consisted in the reduction of the terminal methylene group and the transformation of the second oxygen of veatchine to an hydroxyl group. As this second oxygen was not a carbonyl group, it was assumed that it might be present in the form of an ether or oxide bridge. However, all attempts to cleave such a group resulted in the recovery of starting material.

Reduction of veatchine with lithium aluminum hydride yielded a dihydro compound, in which the carbon–carbon double bond associated with the nitrogen was reduced, as evidenced by the change in the pK value of this derivative from 11.5 to 6.85 (that of tetrahydroveatchine). The dihydro derivative, however, still exhibited the presence of a terminal methylene group in the infrared spectrum, and gave formaldehyde on ozonolysis. These facts prove that there is a methylene group present, which is not associated with the nitrogen, and, therefore, a total of two carbon–carbon double bonds in veatchine and garryine.

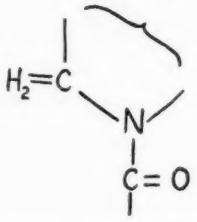
The oxidation of veatchine with potassium permanganate under mild conditions yielded two neutral products (Compound *A* and Compound *B*), both analyzing for $C_{22}H_{31}O_3N$. A difference was apparent not only in their melting points, but also in their infrared spectra. Both compounds are thought to be amides derived from veatchine by the abstraction of two hydrogens and the addition of one oxygen atom. The nature of these compounds becomes obvious upon comparison of their carbonyl frequencies in the infrared region. Compound *A* (m.p. 210°C.) has a carbonyl peak at 1690 cm.$^{-1}$ and in more dilute solution at 1700 cm.$^{-1}$, whereas Compound *B* (m.p. 233°C.) has a carbonyl peak at 1630 cm.$^{-1}$

The wave length of the carbonyl peak of Compound *A* indicates an amide

group in a ring, possibly a six-membered ring condensed with several other rings or a five-membered ring which is not a part of a highly condensed system. The carbonyl group of Compound *B*, on the other hand, is likely to belong to a disubstituted amide, which is not in a ring. The ultraviolet spectra of both compounds show end absorption. It seems, therefore, that the relationship of the two compounds can be represented by the following structures:



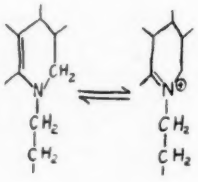
Compound A



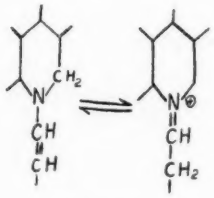
Compound B

The oxidation of garryine with permanganate gives a high yield of a neutral crystalline product $C_{22}H_{33}O_3N$. The empirical formula of this compound shows that only one oxygen has been added to garryine. This fact can be explained in the following manner:

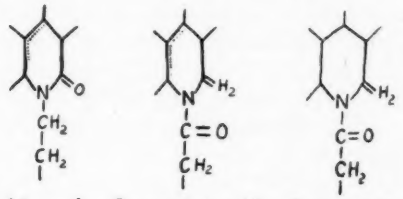
The infrared spectrum of oxogarryine shows a strong band at 1618 cm.$^{-1}$ with a shoulder at 1637 cm.$^{-1}$ This is a wave length comparable with that of oxoveatchine *B*, and very different from that of oxoveatchine *A*, in which the amide group seems to be in a ring. The carbonyl group in oxogarryine can, therefore, be best explained as that of a disubstituted amide, which is not a part of a ring. If we accept that the amide group in oxogarryine is formed from the carbon–carbon double bond, and assume for the present that the ring containing the nitrogen is six-membered, we obtain the following tentative scheme for all the compounds involved:



Veatchine



Garryine



Oxoveatchine A Oxoveatchine B Oxogarryine

This scheme is in agreement with basicities of the compounds involved. It is well known from the work of Adams and Mahan (1) that the basifying influence of a carbon–carbon double bond in vinyl tertiary amines is stronger if the double bond is in a ring than if it is in an open chain.

There is, however, one difficulty which necessitates caution in the interpretation of these reactions. In view of the parallelism of the chemistry of atisine and veatchine (*vide infra*), this scheme, which is based on the infrared carbonyl frequencies of the oxoveatchines and oxogarryine, seems to be in contradiction to the properties and further reactions of the tricarboxylic acid, IV, derived from isoatisine. However, as no infrared spectrum of oxoisoatisine has been published and the corresponding experimental data on acids derived from garryine are not at present available, further development will have to be awaited. A comprehensive study of the infrared spectroscopy of six-membered lactams is also required.

The carbonyl frequency of 1,3-dimethylhexahydrooxindole has been observed at 1690 cm.$^{-1}$, which is identical with that of oxoveatchine *A*. It is also possible that condensation with other rings, in the case of a six-membered lactam, could produce a similar frequency.

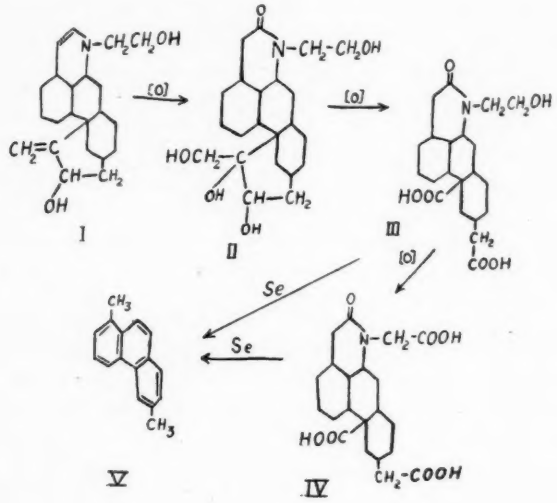
The infrared spectrum of oxogarryine contains two features which are not found in the spectrum of oxoveatchine *B*: one is a band at 1508 cm.$^{-1}$; the other is a double band at 3245 cm.$^{-1}$ and 3368 cm.$^{-1}$ The 1508 cm.$^{-1}$ and 3245 cm.$^{-1}$ bands could, perhaps, be ascribed to N–H bending and N–H stretching of a monosubstituted amide, which is not a part of a ring. However, unless more evidence is produced to support such a view, this appears unlikely.

Finally, we have reduced oxogarryine with lithium aluminum hydride to obtain dihydroveatchine, which is identical, as shown by infrared spectra, with the dihydroveatchine obtained by the action of the same reagent on veatchine. This evidence is, of course, difficult to reconcile with the assumption that oxogarryine is a monosubstituted amide, but it is fully in agreement with the assumption that the amide group originated from the carbon–carbon double bond without any change in the skeleton.

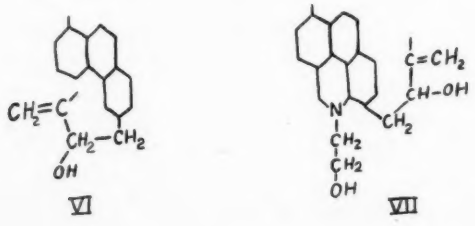
All the reactions of veatchine and garryine are strongly reminiscent of the relationship of atisine and isoatisine, which has been reported in a series of papers by Jacobs and his collaborators (5, 6, 7, 8, 9, 10). The methylenic nature of the double bond in atisine, although assumed, has not been proved. Permanganate

oxidation of atisine did not yield neutral substances analogous to our oxoveatchines *A* and *B*, but acids which were derived from an oxoatisine $C_{22}H_{31}O_3N$.

In his most recent paper, Jacobs proposed the tentative structure I for isoatisine. This structure was based mainly on the isolation of 1-methyl-6-ethyl phenanthrene from the selenium dehydrogenation of atisine and the series of degradations as indicated by the formulae I-V:



From these reactions it is evident that three of the four carbocyclic rings form a perhydrophenanthrene skeleton. The fourth carbocyclic ring can be opened with the formation of a dicarboxylic acid. From the dehydrogenation of this diacid to 1,6-dimethylphenanthrene, one point of attachment of this fourth ring can be located at carbon 6. The second point of attachment is likely to be a tertiary carbon atom. This follows from the properties of one of the carboxyl groups of the dicarboxylic acid. However, the point of attachment in formula I is not the only posssible one. The isolation of the tricarboxylic acid represented by formula IV indicates the primary character of one of the hydroxyl groups in I. The mode of attachment of the nitrogen ring in I is tentative.



In summary, we have, therefore, a partial structure VI fairly well established (although variations in the order of the carbon–carbon double bond and the hydroxyl group in the ring attached to carbon 6 appear possible), and to complete the structure we have only to add, in addition to the N-alkyl group, one carbon and one nitrogen atom to form the nitrogen ring. Finally, one carbon–carbon bond has to be located between the end of the side-chain at carbon 6 and one tertiary carbon of the perhydrophenanthrene skeleton.

The manner in which the nitrogen ring is completed in formula I seems to us to be in contradiction to the results of the Kuhn-Roth determination, which indicates one C-methyl group for both atisine and veatchine.

Another purely tentative way to complete the partial structure is depicted by formula VII. This formula would explain the C-methyl group, and it would be in better agreement with the basic dehydrogenation product.

We have preliminarily reported (3) a base $C_{16}H_{15}N$, which was obtained from veatchine by selenium dehydrogenation just as a similar base was obtained by Jacobs (9) from atisine. Our base has no active hydrogen, no N-alkyl group, and a pK value (80% methyl cellosolve) of 4.0. The ultraviolet spectrum of this base is given in Fig. 9 (Curve 1).

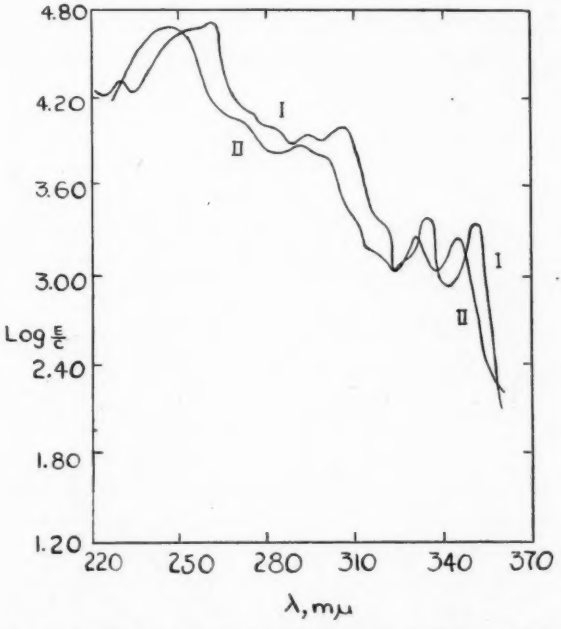


FIG. 9. Ultraviolet spectra. I. $C_{16}H_{15}N$ base. II. Phenanthridine.

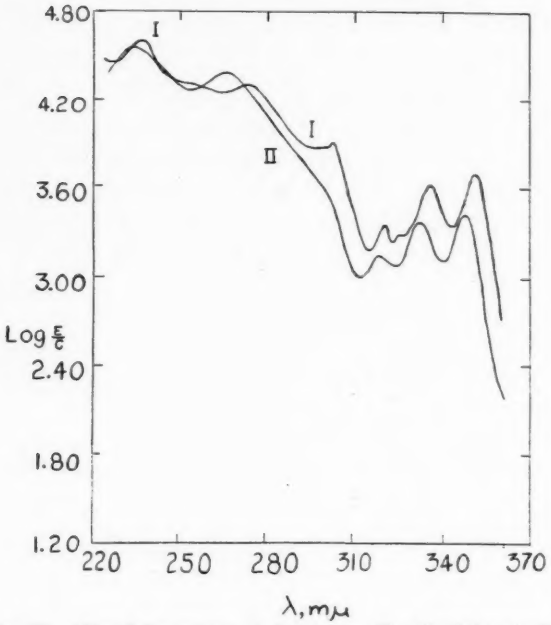


FIG. 10. Ultraviolet spectra. I. Azaretene. II. α-Naphthoquinoline.

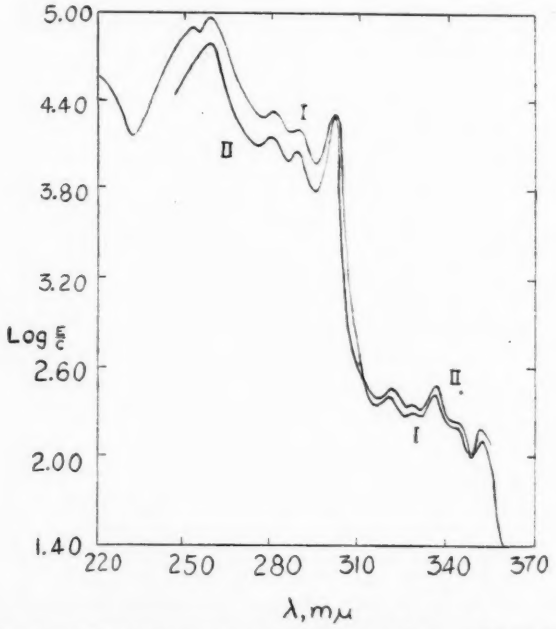


FIG. 11. Ultraviolet spectra. I. $C_{17}H_{16}$ hydrocarbon. II. Pimanthrene.

This spectrum (Fig. 9, Curve 1) is obviously that of an azaphenanthrene. The spectra of different azaphenanthrenes are similar to spectra of phenanthrenes and very different from those of acridines or other heterocycles (2). If we compare, however, the spectrum of our base with that of α-naphthoquinoline (Fig. 10, Curve 2) or azaretene (Fig. 10, Curve 1), we see that, although they are of the same general type, there are undoubtedly differences which cannot be ascribed to substitution. On the other hand, the spectrum of phenanthridine (Fig. 9, Curve 2) is similar in its entirety to that of our base, except that the latter is shifted to a higher wave length. Such a shift to higher wave length is the result of substitution, as can readily be shown by comparing the spectra of substituted and unsubstituted phenanthrenes or those of azaretene and β-naphthoquinoline (4).

We believe that elucidation of the structure of the $C_{16}H_{15}N$ base will clarify considerably the structure around the nitrogen atom; appropriate synthetic and degradative studies are in progress.

From the neutral material produced in the dehydrogenation of veatchine, a phenanthrene hydrocarbon $C_{17}H_{16}$ (m.p. 91.5°C.; m.p. of trinitrobenzene complex 135-136°C.) was isolated. The spectrum of this compound (Fig. 11, Curve 1) is identical with that of retene or pimanthrene (Fig. 11, Curve 2).

The reported melting point (12) of 1-methyl-7-ethyl phenanthrene is 87.5°C. and that of its trinitrobenzene complex is 134°C. We are at present undertaking the synthesis of this compound.* Thus it would seem that the point of attachment of the fourth carbocyclic ring is one of the main differences between the structure of atisine and that of veatchine.

EXPERIMENTAL PART

Isolation of Alkaloids

The crude alkaloid mixture was isolated by a modification of Oneto's method (11). The ground bark was percolated with alcohol, and the percolate was evaporated to a small volume. The residue was dissolved in water, and the solution was filtered. The filtrate was made strongly alkaline with sodium hydroxide and extracted three times with chloroform. The volume of the chloroform extract was reduced *in vacuo*, and the bases were separated by shaking with 5% sulphuric acid. The acidic extract was then made alkaline, and the precipitated bases were extracted with chloroform. The yield of crude bases was 1.1%.

From the mixture of crude bases veatchine and garryine were separated by countercurrent distribution. The crude bases were first distributed between a phosphate–citrate buffer of pH = 7 and chloroform using nine funnels (200 ml. of each phase). A typical experiment employing 23 gm. of the crude mixture gave the following result:

**Note added to proof: This has now been completed and the identity of our phenanthrene with 1-methyl-7-ethyl phenanthrene has been established by mixed melting points and infrared spectra.*

Funnel No.	Wt. of substance (gm.)
1	4.188
2	4.228
3	4.457
4	4.278
5	3.200
6	0.571
7	0.229
8	0.596
9	1.811

Recrystallization of the contents of the first three funnels from acetone–water yielded pure veatchine, which melted at 119-120°C. after six recrystallizations. It was sublimed for analysis in high vacuum at 140°C. Another sample was dried in high vacuum at 80°C.

Calc. for $C_{22}H_{33}O_2N$: C, 76.92; H, 9.68; N, 4.08; act.H, 0.29; (N)–CH_3, 4.37; (C)–CH_3, 4.37%.

Found: C, 76.97, 76.75, 76.75; H, 9.73, 9.84, 9.80; N, 4.19, 4.31, 4.22; act.H, 0.31; (N)–CH_3, 4.17; (C)–CH_3, 2.89%.

Microtitration in methyl cellosolve: pK = 11.5.

The last two funnels of the countercurrent distribution contained garryine, and the contents were redistributed between a phosphate–citrate buffer of pH = 5.5 and chloroform (100 ml. of each phase).

The result was as follows:

Funnel No.	Wt. of substance (gm.)
1	0.146
2	0.154
3	0.220
4	0.243
5	0.297
6	0.191
7	0.093
8	0.120
9	0.718

The material from funnels 3, 4, and 5 yielded 768 mgm. of pure garryine upon recrystallization from acetone–water. After eight recrystallizations the hydrate melted over the range 74-82°C. On warming or drying *in vacuo*, the hydrate lost water and was transformed into the oily anhydrous compound. For analysis it was distilled in a sublimation tube at 140°C. in high vacuum.

Calc. for $C_{22}H_{33}O_2N$: C, 76.92; H, 9.68; N, 4.08; act.H, 0.29; (N)–CH_3, 4.37; (C)–CH_3, 4.37%.

Found: C, 76.92, 76.64, 76.51; H, 9.78, 9.79, 9.53; N, 4.09, 4.41; act. H, 0.27; (N)–CH_3, 2.13; (C)–CH_3, 1.94%.

Microtitration in methyl cellosolve: pK = 8.7.

Veatchine Hydrochloride

This material was recrystallized eight times from absolute alcohol – ether. It melted at 267-271°C. Potentiometric microtitration showed pK = 11.5.

Calc. for $C_{22}H_{33}O_2N.HCl$: C, 69.55; H, 9.02; N, 3.69; Cl, 9.33%.

Found: C, 69.12; H, 9.32; N, 3.46; Cl, 9.48%.

Garryine Hydrochloride

This compound, when recrystallized eight times from absolute alcohol – ether, melted at 263-268°C. Potentiometric microtitration showed pK = 8.7.

Calc. for $C_{22}H_{33}O_2N.HCl$: C, 69.55; H, 9.02; N, 3.69; Cl, 9.33%.

Found: C, 69.44; H, 9.05; N, 3.78; Cl, 9.26%.

Acetyl Veatchine

Veatchine (100 mgm.) was dissolved in 1 ml. of dry pyridine and 2 ml. of acetic anhydride and allowed to react at room temperature for 60 hr. At the end of this period the solution was evaporated to dryness *in vacuo*, dissolved in chloroform, and washed several times with 10% hydrochloric acid. Upon evaporation of the chloroform, crystalline acetyl veatchine hydrochloride remained. It was recrystallized to a constant melting point of 258-259°C. The free base was liberated and recrystallized from acetone–water. The latter compound melted at 152-153°C., and was sublimed for analysis.

Calc. for $C_{24}H_{35}O_3N$: C, 74.77; H, 9.15; acetyl, 11.16%.

Found: C, 75.15, 74.51; H, 8.71, 9.24; acetyl. 9.55%.

Microtitration: pK = 11.5.

Hydrogenation of Veatchine

Veatchine (500 mgm.) was hydrogenated in 20 ml. of glacial acetic acid with 100 mgm. of platinum oxide. The uptake of hydrogen (2 moles) was completed in two-three hours. The reaction mixture yielded tetrahydroveatchine, which was recrystallized six times from acetone–water to a melting point of 147-149°C. It was sublimed for analysis at 120°C. in high vacuum.

Calc. for $C_{22}H_{37}O_2N$: C, 76.00; H, 10.73; N, 4.03; 2 act.H, 0.58; 1 (C)–CH_3, 4.32%.

Found: C, 76.05; H, 10.77; N, 4.11; act.H, 0.55; (C)–CH_3, 6.08, 5.87%.

Microtitration in methyl cellosolve: pK = 6.8.

The picrate of the compound melted at 206-207°C.

Hydrogenation of Garryine

Garryine showed an uptake of 2 moles of hydrogen under the same conditions as were used in the case of veatchine. The resulting tetrahydrogarryine melted at 148-150°C. (acetone–water), and gave no depression upon admixture with tetrahydroveatchine. It was sublimed for analysis.

Calc. for $C_{22}H_{37}O_2N$: C, 76.00; H, 10.73; N, 4.03; 2 act.H, 0.58; (N)–CH_3, 4.32%.

Found: C, 76.02; H, 10.79; N, 4.25; act.H, 0.59; (N)–CH_3, 4.41%.

Tetrahydrogarryine picrate melted at 205-207°C. (alcohol), and did not depress the melting point of tetrahydroveatchine picrate. The former picrate was dried at 80°C. for 24 hr. in high vacuum and then analyzed.
Calc. for $C_{22}H_{37}O_2N.C_6H_3O_7N_3$: C, 58.24; H, 7.04; N, 9.71%.
Found: C, 58.41; H, 6.92; N, 9.94%.

Diacetyl Tetrahydroveatchine

Tetrahydroveatchine (500 mgm.) was acetylated in the usual way with pyridine and acetic anhydride at room temperature. The reaction mixture was evaporated to dryness and the residue dissolved in chloroform. The chloroform solution was then washed with 5% hydrochloric acid and water and evaporated to dryness. The residue contained the crude gummy hydrochloride of diacetyl tetrahydroveatchine. This was again dissolved in chloroform, and the free base was liberated by washing the solution with dilute alkali. Purification of this compound was achieved by chromatography on neutral alumina in absolute benzene, followed by distillation in a sublimation tube at 140°C. in high vacuum. Potentiometric microtitration in methyl cellosolve showed pK = 5.6.

Calc. for $C_{26}H_{41}O_4N$: C, 72.35; H, 9.58; acetyl, 20.00%.
Found: C, 72.37; H, 9.72; acetyl, 21.65%.

Ozonolyses

Veatchine, garryine, dihydroveatchine, and tetrahydroveatchine (200 mgm. of each) were ozonized in absolute chloroform at 0°C. for three hours. The chloroform was evaporated from each *in vacuo* at room temperature, and 5 ml. of 5% sulphuric acid was added. The mixtures were then steam-distilled, and 2 ml. of 5% ethanolic dimedone solution was added to each distillate. Garryine, veatchine, and dihydroveatchine gave a positive result; approximately 0.5 mole of crystalline dimedone–formaldehyde complex was obtained in each case (m.p. 183-186°C. and no depression upon admixture with an authentic specimen). Tetrahydroveatchine gave only traces of precipitate.

Conversion of Veatchine to Garryine

Veatchine (1 gm.) was refluxed for three hours with 50 ml. of 5% methanolic potassium hydroxide. The alcohol was distilled *in vacuo* and water was added. The resulting precipitate was extracted with chloroform, and this solution was evaporated to dryness. The residue was distributed in nine funnels between a phosphate–citrate buffer of pH = 7 and chloroform (50 ml. of each phase). The result was as follows:

Funnel No.	Wt. of substance (gm.)
1	0.08
2	0
3	0
4	0
5	0.005
6	0.038
7	0.158
8	0.386
9	0.360

The contents of funnels 6, 7, 8, and 9 were garryine. It was recrystallized several times from acetone–water and melted at 75-82°C. It was then sublimed for analysis.

Calc. for $C_{22}H_{33}O_2N$: C, 76.92; H, 9.68%.
Found: C, 77.03; H, 9.69%.
Microtitration in methyl cellosolve: pK = 8.7.

Dihydroveatchine

Veatchine (200 mgm.) was placed in a Soxhlet and extracted overnight with 30 ml. of absolute ether containing 1 gm. of lithium aluminum hydride. The excess hydride was destroyed with water, and the mixture was worked up in the usual manner. The product crystallized from acetone–water and melted at 141-143°C. No depression of melting point was evidenced in admixture with tetrahydroveatchine. However, the infrared spectrum distinctly showed several differences, one of these being the presence of a band assigned to a $C = CH_2$ group in veatchine; this band is absent in the spectrum of tetrahydroveatchine.

Calc. for $C_{22}H_{35}O_2N$: C, 76.47; H, 10.21%.
Found: C, 76.47; H, 10.24%.
Microtitration in methyl cellosolve: pK = 6.9.

The picrate of this compound melted at 193-195°C. (methanol).

Permanganate Oxidation of Veatchine

Veatchine (5 gm.) was dissolved in 335 ml. of acetone and 6 gm. of potassium permanganate in 260 ml. of acetone was added dropwise at 10-15°C. The reaction was allowed to proceed at this temperature for four and one-half hours. Manganese dioxide was removed by filtration, and evaporation of the acetone yielded 2.49 gm. of product. This was separated into a neutral fraction (493 mgm.) and a basic fraction (unchanged veatchine).

The manganese dioxide was suspended in a mixture of water and chloroform (200 ml. of each), and the whole was cooled to 5°C. Sulphur dioxide was then passed into the mixture until all the manganese dioxide was reduced. The chloroform layer was separated, and the aqueous layer was extracted several more times with chloroform. The combined chloroform layers yielded 1.08 gm. of white foam. This was combined with the neutral material obtained previously (493 mgm.), and the whole was separated into a neutral fraction (698 mgm.) and an acidic fraction.

Chromatography of the neutral material on alumina yielded two main substances: *Compound A*, 133 mgm. of crystalline material eluted with absolute benzene, and *Compound B*, 162 mgm. of white foam eluted with absolute ether. Compound *A* melted at 211-213°C. and Compound *B* at 234-237°C.; both were recrystallized from absolute methanol – ether. A mixture of the two compounds melted at 160-200°C. That the two compounds were distinctly different was further shown by the differences in their infrared spectra, especially in the carbonyl region.

Calc. for $C_{22}H_{31}O_3N$: C, 73.91; H, 8.76; N, 3.92%.
Found for Compound *A*: C, 73.88, 73.82; H, 8.45, 8.65; N, 3.85%.
Found for Compound *B*: C, 74.19, 74.20; H, 8.85, 8.63; N, 3.60%.

Permanganate Oxidation of Garryine

Garryine (500 mgm.) was dissolved in 50 ml. of acetone and 0.5 ml. of glacial acetic acid. To this solution 300 mgm. of finely powdered potassium permanganate was added over a period of 30 min. The stirring was continued for a further 20 min. The residue upon separation yielded 268 mgm. of neutral material and a small amount of unchanged garryine. The neutral material was recrystallized from ethyl acetate to a constant melting point of 187-188°C.

Calc. for $C_{22}H_{33}O_3N$: C, 73.48; H, 9.26; N, 3.90%.
Found: C, 73.60, 73.38; H, 9.32, 9.11; N, 3.71%.

Conversion of Oxogarryine to Dihydroveatchine

Oxogarryine (500 mgm.) was reduced with 1.5 gm. of lithium aluminum hydride in 50 ml. of absolute ether using a Soxhlet extractor. After being worked up in the usual manner, the product was recrystallized from acetone–water (m.p. 148°C.). This substance did not depress the melting point of dihydroveatchine, and its infrared spectrum was identical with that of dihydroveatchine. A sample was sublimed for analysis in high vacuum.

Calc. for $C_{22}H_{35}O_2N$: C, 76.47; H, 10.21%.
Found: C, 76.43; H, 10.20%.

Dehydrogenation of Veatchine

Veatchine (20 gm.) was mixed with 40 gm. of red selenium, and the whole was heated in an atmosphere of nitrogen at 340°C. for 12 hr. The selenium "cake" was pulverized and extracted overnight in a Soxhlet with ether. The total amount of a brown-colored viscous oil thus obtained was 15.12 gm. This was separated into four fractions by extracting in the usual manner with hydrochloric acid, sodium bicarbonate, and sodium hydroxide. The fractions were:

1. Neutral, ether soluble; 9.38 gm.
2. Neutral, chloroform soluble; 1.87 gm.
3. Basic; 3.75 gm.
4. Phenolic; 0.124 gm.

Chromotography of the Basic Fraction

The basic material was chromatographed on 150 gm. of untreated Fisher alumina, 250 ml. fractions being taken. The result was as follows:

1. Fractions 1-8 eluted with absolute benzene yielded 0.409 gm. of colorless oil.

2. Fractions 9-26 eluted with absolute benzene yielded 1.047 gm. of crystals, m.p. 65-95°C.

3. Fractions 27-36 eluted with absolute benzene – ether (1 :1) yielded 0.927 gm. of crystalline material.

4. Further fractions eluted with absolute ether – chloroform and absolute methanol – chloroform were dark and resinous and have not yet been investigated.

The crystalline material was recrystallized to a constant melting point of 115°C. (absolute ether). This compound was then sublimed at 90°C. in high vacuum for analysis.

Calc. for $C_{16}H_{15}N$: C, 86.83; H, 6.84; N, 6.33%.
Found: C, 87.20, 87.33; H, 6.83, 7.01; N, 6.07, 5.99; (N)–CH_3, 0.0; act.H, 0.0%.
Microtitration in methyl cellosolve: pK = 4.0.

The picrate of this base melted at 243°C. It was dried at 80°C. for 24 hr.

Calc. for $C_{16}H_{15}N.C_6H_3O_7N_3$: C, 58.64; H, 4.03; N, 12.44%.
Found: C, 58.98, 58.93; H, 4.02, 3.80; N, 12.83%.

Chromatography of the Neutral Fractions

The neutral material was chromatographed on 500 gm. of untreated Fisher alumina, 500 ml. fractions being taken. The result was as follows:

1. Fractions 1-6 eluted with absolute petroleum ether yielded 1.261 gm. of an oily liquid.

2. Fractions 7-19 eluted with absolute petroleum ether yielded 2.48 gm. of crystalline material.

3. Further fractions eluted successively with benzene, ether, and methanol–chloroform were oily and have not yet been investigated.

Several samples of the crystalline material were prepared by successive recrystallization from petroleum ether, ether, and finally methanol. All samples melted at 91.0-91.5°C., and they were sublimed at 80°C. in high vacuum.

Calc. for $C_{17}H_{16}$: C, 92.68; H, 7.32%.
Found: C, 92.38, 92.59, 92.55, 92.62; H, 7.33, 7.36, 7.31, 7.23%.

The trinitrobenzene complex melted at 135-136°C. (alcohol). A sample was dried for analysis at 40°C. in high vacuum for 48 hr.

Calc. for $C_{17}H_{16}.C_6H_3N_3O_6$: C, 63.71; H, 4.42; N, 9.73%.
Found: C, 64.17; H, 4.23; N, 9.71, 9.80%.

The microanalyses were performed partly by Dr. Robert Dietrich of Zurich and partly in the microanalytical laboratory of the University of Pittsburgh.

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